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Effects of herbicides on mitochondrial enzyme systems

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NOTE TO USERS

EFFECTS OF HERBICIDES ON MITOCHONDRIAL ENZYME SYSTEMS

by

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INTRODUCTION

The use of chemicals for weed control is widespread, but knowledge concerning the mechanism of action of many of these herbicides is meagre or lacking. Investigations of this problem have dealt mainly with intact plants or plant parts such as segments of stems, roots, and coleoptiles. With such material it is almost impossible to pin-point the site of action of a herbicide because of the interacting effects of the cell membrane, the cytoplasm, and the many enzyme systems contained therein. In vitro study of the response of isolated enzyme systems to herbicides would be expected to be more rewarding. Such a study is the subject of this dissertation.

Although herbicides have been shown to affect many diverse processes in plants, respiratory responses are general and the idea that a common site of action exists somewhere in the oxidative or phosphorylative systems would seem to be a good working hypothesis. In recent years evidence has accumulated that the enzymes involved in aerobic respiration and phosphorylation are located in discrete cytoplasmic particles called mitochondria. Each mitochondrion apparently contains the entire complement of enzymes and coenzymes necessary for the total oxidation of pyruvate and other intermediates of the Krebs cycle to carbon dioxide and water. The energy thus liberated is used in the

formation of high-energy phosphate bonds. These reactions may be carried out in vitro by properly isolated mitochondria. The complex of enzymes associated with such mitochondria is probably closer to its natural condition in the plant than individual enzymes in cell-free preparations could be expected to be. Thus the effect of a herbicide on isolated mitochondria would probably be indicative of the way in which the herbicide would affect mitochondrial enzymes in vivo. However, the possibility that the mitochondrial system has been changed in some manner during isolation and therefore reacts differently must be kept in mind.

The object of this study was to investigate the properties of soybean mitochondria and the effects of various herbicides upon certain activities of these particles. Soybeans were chosen for investigation; first because the effect of herbicides on intact plants has been well characterizied; second, although the plant is related to others from which mitochondria have been isolated, the mitochondria of soybeans had not been investigated. Information concerning the properties and activities of mitochondria from a wide variety of plants is necessary before it can be established whether or not particles from all plants behave in a similar manner.

There has been some discussion in the literature of the proper terminology for use in studies involving the biochemical reactions of cytoplasmic particles. Stafford (71) and Laties (40) have pointed out that differential centrifuga-

tion may bring about good separation of biochemical systems without producing microscopically uniform fractions. The cytologist would probably not use the term mitochondria for the components of such a heterogeneous fraction (57). In the physiological literature, where authors have been primarily interested in the biochemical properties of a given fraction, the terms mitochondria and particles have often been used interchangeably (40). This procedure will be followed throughout the present study.

GLOSSARY

Abbreviations as listed below will be used throughout this dissertation.

AMP	Adenosine-5-phosphate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Co A	Coenzyme A
Cyt. c	Cytochrome c
2,4-D	2,4-Dichlorophenoxyacetic acid
DCP	2,4-Dichlorophenol
DCPA	2,2-Dichloropropionic acid
DNP	2,4-Dinitrophenol
DPN	Diphosphopyridine nucleotide
IAA	Indoleacetic acid
Mu	Micron
$Q_{O_2}(N)$	Net oxygen uptake (total minus endogenous) in microliters/hr/mg nitrogen.
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
TCA	Trichloroacetic acid
TIBA	2,3,5-Triiodobenzoic acid
Tris.	Trishydroxymethylaminomethane

LITERATURE REVIEW

Mitochondria

Cytology, morphology, and chemical composition of mitochondria

The cytological literature on plant mitochondria has been carefully reviewed by Newcomer (59). The name mitochondria was proposed for small cytoplasmic particles by Benda in 1898 (Millerd and Bonner, 52) from "mitos" meaning thread, and "chondrione" meaning a small grain. The term microsomes was often used interchangeably with mitochondria in the early literature. Now, however, microsomes are considered to be smaller particles than mitochondria and to have different chemical composition and biological activities.

The occurrence of mitochondria in plant tissues was first demonstrated by Meves in 1904 (Millerd and Bonner, 52). They may occur in various shapes from rod-like to spherical, and in various sizes from less than 1 mu up to 12 mu in length. A range of 1 to 4 mu in length is considered usual for rod-like particles by Millerd and Bonner (52). Rods from 2 to 6 mu in length, and spheres from 0.1 to 6.0 mu in diameter were observed by Stafford (71).

The studies of Palade (60, 61), using the electron microscope, show clearly that mitochondria are bounded by a membrane. This is in contrast to the conclusions of Harmon (33) that animal mitochondria have a protein gel structure and no limiting membrane. The opinion that a membrane is

present which controls the passage of substances, separates enzymes from their substrates, and to some degree influences enzymatic reactions, is widely held at the present time (46).

Newcomer (59) has discussed the problem of mitochondrial morphology. It is possible that there may be more than one type of particle, or that all particles are the same but some of them (proplastids) develop into plastids while others remain as mitochondria. Because of this problem, Goddard and Stafford (27) have suggested that mitochondria be defined in terms of enzymatic activity. They define mitochondria as "cellular particles associated with enzymes of the cytochrome system, the Krebs cycle, fatty acid oxidation, and with oxidative phosphorylation." The staining of mitochondria with Janus green B has been widely used as a criterion for active particles (20, 53, 68). The staining is probably brought about by the specific re-oxidation of the dye by cytochrome oxidase, which is found only in mitochondria. Lazarow and Cooperstein (44) studied the enzymatic mechanism involved in Janus green B staining reaction. They concluded that although this dye is specific for mitochondria in intact tissue, it is not necessarily so for isolated particles. Newcomer (59) and Stafford (71) were unable to see this stain in isolated particles. Stafford (71) found no dye that would stain particles from pea seedlings selectively or without bringing about clumping. In addition to Janus green B, the Nadi reagent, tetrazolium

salts, methyl green, and pyronin were tested. It would appear that some caution should be exercised in the use of any of these stains for the identification of mitochondria in homogenates.

The principal chemical constituents of mitochondria are lipids and proteins. Over 90 per cent of the dry weight of animal mitochondria has been shown to be made up of lipids, proteins, and ribose nucleic acids (46). Stafford (71) found that mitochondria isolated from peas contained 30 to 40 per cent protein, 25 to 38 per cent lipids, 0.5 to 1.0 per cent pentose nucleic acid, and 0.7 to 0.9 per cent desoxypentose nucleic acid on a dry weight basis. The latter constituent was presumed to be a contaminant from nuclear destruction during preparation.

Mitochondrial enzymes

Newcomer (59) credits Kingsley in 1912 as being the first to suggest that respiratory enzymes are located in (or on) mitochondria. Keilin (37) described the isolation of particles containing succinate and cytochrome oxidase. He is credited by Lindberg and Ernster (46) as being the first "to isolate an integrated multi-enzyme system."

The problem of establishing the exact cellular localization of various enzymes is a difficult one. Schneider (66) has pointed out that mitochondrial preparations may be contaminated with other parts of the cell and thus appear to have activities not actually possessed by the mitochondria

in vivo. Schneider and Hogeboom (67) proposed a criterion for establishing the presence of an enzyme in a given cell fraction. This criterion is that the particular enzyme must be more concentrated in the given fraction than in the cell as a whole. Enzymes considered to be present in animal mitochondria by this criterion are cytochrome and succinic oxidases, oxaloacetate and octanoate oxidases, DPN-cytochrome c reductase, and the system which synthesizes p-aminohippuric acid. Many others have been found in isolated particles, but satisfactory data concerning their relative concentration have not as yet appeared in the literature (46).

The enzymes and coenzymes necessary for the complete oxidation of pyruvic acid to carbon dioxide and water are probably held in a precise structural relationship. This enzyme system has been called the cyclophorase system by Green *et al*. (30). It is pictured as an integrated multienzyme system very different from a random mixture of its constituent enzymes. Harmon (33) has suggested that mitochondria represent the structural units of the cyclophorase complex. This was contested by Schneider and Hogeboom (67), but Lindberg and Ernster (46), in their comprehensive review of the mitochondrial literature, accept the hypothesis that the cyclophorase system is associated with the mitochondria. They point out, however, that the two systems are not equivalent or the two terms interchangeable. The cyclophorase system is an integral part, but only

a part, of the mitochondrion.

Hill and Bhagvat (34) were the first to show that succinoxidase was associated with the insoluble particles of the cytoplasm of plant cells. Stafford (71) found cytochrome oxidase and succinic oxidase activities on particles isolated from etiolated seedlings of Pisum sativum. Although these particles differed in shape from typical rod-shaped plant mitochondria, Stafford believed that they were functionally similar to mitochondria isolated from animal tissues. Millerd et al. (53) were the first to show that cytoplasmic particles from a higher plant could completely oxidize pyruvate to carbon dioxide and water. No cofactors were required other than a catalytic amount of one of the acids of the Krebs cycle, although the rate of oxidation was increased by the addition of ATP and magnesium ions. These particles were able to oxidize all the principal acids of the Krebs cycle. For all acids tested, substrate saturation was essentially reached at a concentration of 2×10^{-2}M (51).

Mung bean particles contained an ATPase which complicated the detection of ATP synthesis (53). This ATPase was inhibited with sodium fluoride, and these workers were able to show, by adding isotopically labelled inorganic phosphate to the reaction mixture, that the energy liberated in (∝-) ketoglutarate oxidation was utilized in the formation of energy-rich phosphate bonds of ATP. Further work on the phosphorylative capacity of particles isolated from mung

beans (16) and from avocado fruits (54) indicated that about one mole of phosphate was esterified for each atom of oxygen consumed (P:O ratio = 1). Laties (42), however, using particles isolated from cauliflower buds, has obtained P:O ratios of 2 for succinic oxidation and 3 for malic and ketoglutaric acid oxidation. He suggests that even higher P:O ratios for ketoglutaric acid oxidation may occur since, at the time of his measurements, part of the phosphorylative system was already inoperative. Laties (42) postulates further that the P:O ratios of 1 obtained by Bonner and Millerd (16) were possibly due to greater loss of activity during preparation of mung bean mitochondria than was the case with particles prepared from cauliflower.

Further evidence that the Krebs cycle complex of enzymes is localized in the cytoplasmic particles of plant cells has been provided by the work of Dow (22) with white lupine seedlings, Davies (20) with etiolated pea seedlings, Hackett and Simon (32) with the spadix of Arum maculatum, and Tager (72) with Avena seedlings. Most of these investigations have been carried out with etiolated tissue, but McClendon (49) has shown that cytochrome oxidase in green leaves of tobacco is localized in small particles, presumably mitochondria.

Very few data concerning carbon dioxide evolution during mitochondrial respiration have appeared in the literature. Millerd et al. (53) found that the respiratory quotient

(r.q.) for the oxidation of pyruvate by mitochondria from mung beans was 1.3, which corresponds to the expected value if pyruvate was being completely oxidized to carbon dioxide and water. Hackett and Simon (32) showed that the r.q. for citrate oxidation was 1.30, for ketoglutarate 1.15, and for succinate 0.38, using particles isolated from the spadix of Arum maculatum. They point out that such values for ketoglutarate and succinate would indicate that these substrates are being converted to fumarate and malate.

Saltman (64) investigated the enzyme hexokinase in potato tuber tissue and concluded that most of it is probably associated with the mitochondria. He showed that magnesium ions were necessary for plant hexokinase activity and that glucose was phosphorylated most rapidly of several hexoses investigated.

Bonner and Millerd (16) state that hexokinase is firmly bound to plant mitochondria and that mitochondria also possess some invertase activity. Thus it was found that even though no exogenous hexose was added to the reaction mixture, hexose monophosphates were formed from ATP generated during the experiment when sucrose and AMP were present. The adequacy of the hexokinase-trapping system of mung bean mitochondria in measurement of phosphorylation was demonstrated by adding an excess of purified yeast or wheat-germ hexokinase. The P:O ratio of ketoglutarate oxidation was not significantly changed by this addition. Laties (40),

on the other hand, found insufficient hexokinase and ATPase present in cauliflower bud mitochondria to permit maximum rates of respiration *in vitro*. Appreciable stimulation of respiration was obtained by addition of glucose and hexokinase.

Other enzymes and enzyme systems have been demonstrated in plant mitochondria. Laties (40) suggested that a diphosphopyridine nucleotide pyrophosphatase is present, because DPN can replace ATP in the reaction mixture. Millerd and Bonner (52) have found both a condensing enzyme and an acetate activating enzyme that play roles in the oxidation of acetate. The activating enzyme is dependent on coenzyme A and ATP and is maintained in the active form by glutathione.

Oxidation of certain amino acids by particle preparations from Avena has been demonstrated by Rautenen and Tager (62). They were not able to show transamination reactions in these systems, and concluded that l-glutamate and l-proline were probably oxidized by specific oxidases. Wilson *et al.* (78), however, found that all amino acids that they tested, except cystine, served as amino donors to ketoglutaric acid in the presence of lupine particles. Apparently transaminases were present on these mitochondria.

Preparation of active mitochondria

The process of disrupting a cell is a drastic one, and the possibility of artifacts must be kept in mind. The problems involved in the isolation of animal mitochondria

are discussed by Schneider and Hogeboom (67). The Potter-Elvehjem homogenizer was found best for rupturing liver cells, and is widely used for this purpose in place of the more violent action of the Waring Blendor. The medium in which the cells are broken has been found to be extremely important. If distilled water is used, the particles swell and their biochemical properties are changed. Isotonic salt solutions often give non-uniform, contaminated, and damaged particles. The use of hypertonic or isotonic sucrose allows isolation of morphologically unchanged mitochondria (46). After disruption of the cells, the homogenate is centrifuged at low speed to sediment nuclei, cell debris, and any unbroken cells. The supernatant is then spun at high speed and the mitochondria obtained as a pellet which may be washed by re-suspension and high-speed centrifugation.

Similar procedures are used for isolation of plant mitochondria. Millerd *et al.* (53) state that particular attention must be given to osmotic concentration, pH, and phosphate concentration of the dispersion medium, and that the temperature must be maintained close to 0°C. Laties (42) studied the effect of changing the physical environment of mitochondria isolated from heads of cauliflower on their oxidative and phosphorylative abilities. He found that the malic, ketoglutaric, and succinic oxidase systems were rapidly and irreversibly damaged by swelling of the mitochondria in hypotonic solution if this occurred in the presence of certain

soluble components of the protoplast. The suspension of mitochondria in distilled water did not affect the succinoxidase system if other protoplast components were absent, but malic and ketoglutaric oxidase systems suffered considerable injury. The presence of dilute solutions of neutral salt minimizes this injury, as does the use of hypertonic solutions of sucrose or mannitol containing dilute (0.01 M) phosphate buffer (52). Any exposure to hypotonic medium completely destroys the phosphorylative system associated with the succinoxidase complex, regardless of the presence of neutral salts. The phosphorylative machinery associated with malic and ketoglutaric oxidation, however, is affected by hypotonic environment to about the same extent as the oxidative systems, and both the oxidative and phosphorylative systems are protected by neutral salt. Laties (42) suggests from these observations that at least part of the phosphorylation accompanying oxidation of malate or ketoglutarate may occur at loci which are not identical with the phosphorylative loci of the succinoxidase system.

The composition of the medium in which the plants are ground is of particular importance (42, 51). Millerd (51) tested various concentrations and proportions of sucrose and phosphate in the grinding medium. Of the various combinations tested, it was found that a sucrose concentration of 0.4 M and a phosphate concentration of 0.1 M yielded particles of highest activity in pyruvate oxidation. The

presence of phosphate seemed to be essential for the isolation of particles possessing maximal oxidative activity. Phosphate also participates in the oxidative reaction. Millerd's experiments (51) indicated that phosphate in the grinding medium acted to inhibit phosphorylytic breakdown of some essential substance or substances. In this respect phosphate could be replaced by other phosphatase inhibitors such as sodium fluoride. Fluoride, however, could not completely replace phosphate, although lower concentrations of phosphate could be used with fluoride than were necessary if no fluoride was added. Apparently phosphate must be present in all stages of the procedure of isolation and use of mung bean mitochondria. This was not due primarily to the action of phosphate as a buffer, since mitochondria prepared in 0.1 M trishydroxymethylaminomethane yielded particles incapable of oxidizing pyruvate. Laties (42), however, found no effect of adding phosphate to sucrose in the grinding medium in which particles were isolated from cauliflower buds. Tager (72) isolated active mitochondria from Avena coleoptiles, using only sucrose in the grinding medium, but Hackett and Simon (32) followed the procedure of Millerd et al. (53) in isolating active particles from the spadix of Arum maculatum. Apparently the necessity for added phosphate is influenced by the plant source of the mitochondria, whereas sucrose or mannitol in hypertonic concentrations must be present in all preparations.

After sedimenting the mitochondria by high speed centrifugation (10,000 times gravity), Millerd et al. (53) washed the particles by resuspension in a solution of sucrose plus phosphate, followed by high speed centrifugation. This technique of washing has subsequently been used by Dow (22), Bonner and Millerd (16) and Laties (42). Millerd (51) showed that this washing procedure lowered endogenous oxygen consumption and increased the activity of mung bean mitochondria toward pyruvate. Tager (72) found, however, that washing Avena coleoptile particles had no effect on pyruvate oxidation, although endogenous oxygen uptake was slightly reduced. In general, it would appear that washing is usually necessary to remove inhibitors or interfering substances from isolated plant mitochondria.

Effect of cofactors on mitochondrial respiration

Laties (40) has demonstrated that mitochondria obtained from cauliflower buds have a primary requirement for adenylate for maximum oxidative activity. The adenylate apparently is necessary for the maintenance of the integrity of the phosphorylative system of the mitochondria at the substrate level, and also it is required as a high energy phosphate acceptor at all levels. Dinitrophenol may be able to replace adenylate by uncoupling phosphorylation from oxidation. No increase in respiration was brought about by dinitrophenol in the absence of adenylate. Adenylate can apparently be equally well supplied by ATP, ADP, or AMP (adenosine-5-phosphate) but not by adenosine-3-phosphoric

acid (53). ATP and DPN are interchangeable with respect to ketoglutaric and succinate oxidation, but not with respect to malate oxidation (49). In the latter case DPN also apparently acts as coenzyme for malic dehydrogenase. Tager (72) found that fumarate oxidation by Avena particles could be increased by increasing DPN concentration from 10^{-4} M to 5×10^{-4} M. He attributed this gain to a possible shift toward malate of the fumarate $\leftrightharpoons$ malate equilibrium as a result of more rapid removal of malate in the DPN-dependent, malic dehydrogenase reaction. Laties (40) showed that the effects of ATP and DPN were neither additive or synergistic, and that adenine, adenosine, or TPN were also effective in bringing about stimulation.

Millerd *et al.* (53) found that the addition of magnesium ions to the reaction mixture markedly increased the rate of pyruvate oxidation. Similar results were obtained with other Krebs cycle acids (51). In cases in which oxidation was increased by both ATP and magnesium used alone, the increase was more than additive when both were present. The addition of magnesium ions and ATP to the reaction mixture seems to have become part of the usual procedure in investigations using plant mitochondria (22, 32, 40, 41, 42, 51, 52, 72).

Mung bean particles, as prepared by Millerd *et al.* (53), showed no response to added cytochrome c. If phosphate was omitted from the grinding medium, however, particles were obtained which did respond to this compound. Laties (40,

41, 42) added cytochrome c routinely to homogenates of cauliflower bud particles, as did Beaudreau and Remmert (6) to kidney bean mitochondria. The rate of oxidation of pyruvate by particles from Avena coleoptiles was not changed by the addition of cytochrome c (72). The species of plant from which mitochondria are isolated as well as the method of isolation is probably important in determining the requirement for exogenous cytochrome c.

Glutathione in the reduced condition has been shown by Beaudreau and Remmert (6) to increase oxygen uptake by particles isolated from kidney bean seedlings. It was considered to be acting as a stabilizer or cofactor. These investigators concluded that added ATP, DPN, TPN, glutathione, phosphate, and manganese ions were necessary for maximum activity of their kidney bean particle system.

Effect of Herbicides and Related Chemicals on Plant Respiration

Indoleacetic acid (IAA)

Early experiments on the respiratory response of plant tissues to auxins were carried out by Bonner (12) in 1933. He obtained stimulation of respiration of Avena coleoptile segments by a crude hormone from Rhizopus suinus. Later, with a purified auxin, Bonner (13) was not able to repeat this observation of respiratory stimulation. Commoner and Thimann (19) observed stimulation of respiration of Avena

coleoptile segments in the presence of the four-carbon acids malic and fumaric. Although they were not able to duplicate this effect of malate on auxin-induced respiration, Berger *et al.* (11) found that the respiration of intact Avena coleoptile tissue was stimulated about 35 per cent in the presence of 10 mg/l of IAA. Such stimulation of respiration of intact tissue has been observed by many investigators since that time (5, 45, 70).

Berger and Avery (8, 9, 10) studied the effects of IAA on enzyme systems of Avena coleoptiles. The activity of both malic and alcohol dehydrogenase was stimulated in coleoptiles treated with auxin. *In vitro* treatment with 0.0025 to 1000 mg/l IAA had no effect on the above two enzymes or on glutamic or isocitric dehydrogenase. Glutamic dehydrogenase activity in these cell-free preparations was inhibited by concentrations of auxin above 1000 mg/l.

Miller and Burris (50) measured the effects of various growth substances upon the oxidation of ascorbic and glycolic acids by cell-free enzymes from barley. IAA had no effect at 0.0001 M but inhibited both oxidations 30 to 50 per cent at 0.0012 M and 0.0125 M. In a similar study by Wagenknecht *et al.* (76), using cell-free enzymes from bean plants, there was little response to IAA, even at a concentration of 0.0125 M.

Bonner (14) has suggested that IAA, adenylic acid, and arginine may promote respiration by functioning in phosphate transfer. In a recent review, Bonner and Bandurski (15)

pointed out that the capacity of the phosphorylating system normally limits the rate of respiration of the Avena coleoptile. Since auxin increases this respiration, they concluded that auxin must be affecting the phosphorylative process. The auxin was believed to mediate an energy transfer toward growth and thus to increase the supply of high-energy-phosphate acceptor sites.

French and Beevers (25) have suggested a more indirect connection between respiration and growth. They proposed that respiratory stimulation by applied auxin results from, rather than precedes, the growth reaction. Auxin was thought to increase growth, resulting in utilization of high-energy phosphate bonds. Respiration could therefore proceed faster because of the presence of more phosphate acceptors.

2,4-D and 2,4,5-T

Brown (18) measured the effect of 2,4-D on the respiration of intact bean plants. He found that carbon dioxide evolution was increased by spraying the plants with 0.1 per cent 2,4-D. Respiration of all treated plants was stimulated for 24 hours, and on a dry weight basis carbon dioxide output from these treated plants remained higher for 4 days. On a per plant basis, however, there was no difference after the second day.

The respiration of slices cut from bean plants treated with 0.1 per cent (1000 ppm) 2,4-D was compared with similar slices from untreated plants by Smith (69, 70). On a dry

weight basis the oxygen uptake of the treated tissue was higher after 24 hours and continued higher for nine days, reaching a maximum by the seventh to ninth day. On a total nitrogen basis, however, the respiration of the treated tissue decreased over this same period of time in relation to that of the control. In experiments in which slices were treated *in vitro* with 2,4-D at concentrations from 0.1 to 100 ppm, it was found that the highest concentration inhibited oxygen uptake about 80 per cent (70). No significant effects were brought about by 0.1 ppm and results with 10 ppm were variable. Similar studies with bean root tissue were carried out by Mitchell *et al.* (55). They found that 2,4-D was inhibitory at concentrations above 10^{-4}M (22 ppm) but slightly stimulatory at 10^{-6}M. Response to growth substances was found to be greater at pH 5 than under less acid conditions, and respiratory inhibition appeared to be proportional, within limits, to the logarithm of the growth substance concentration.

Hsueh and Lou (35) compared the effects of 2,4-D on the germination of rice, an anaerobic seed, and barley, which is aerobic. Rice was found resistant to 1000 ppm of the herbicide, whereas barley germination was completely inhibited by about one-half this concentration. Carbon dioxide evolution appeared to be less inhibited than oxygen uptake. On the basis of these results the authors suggested that the aerobic phase of respiration is more sensitive to 2,4-D than the

anaerobic phase. Similar results have been obtained by other workers, both with higher plants and bacteria (70).

A difference in the sensitivity of the respiratory system of representative dicot (pea) and monocot (oat) plants to 2,4-D was shown by Kelly and Avery (38). They found that oxygen uptake by pea stems was stimulated by concentrations as low as 10^{-4} ppm, whereas a similar stimulation in oat coleoptiles required at least 1000 times this much. No marked toxic effect of 2,4-D on either tissue was noted until a concentration of 1000 ppm was reached. In experiments in which alcohol, malic acid, succinic, and fumaric acids were added to the basal substrate, respiration was found to be slightly stimulated in each case. When 2,4-D (20 ppm) was added, a stimulatory response that was more than additive was produced only in the presence of malate. The authors suggested that this result might indicate a close relationship between 2,4-D stimulation and malic oxidation.

Miller and Burris (50) studied the effects of plant growth substances upon the oxidation of ascorbic and glycolic acids by cell-free enzymes from barley. A similar comparative study was carried out with beans (76). In experiments with barley, 2,4-D and 2,4,5-T were 90 to 100 per cent inhibitory at 10^{-4} M. Ascorbic acid oxidation was unaffected at 10^{-4} M, but inhibited 30 to 50 per cent at 1.2×10^{-3} M and 1.25×10^{-2} M. The cell-free enzymes from bean plants that were tested seemed more resistant than those from barley. The highest concentrations of 2,4-D and 2,4,5-T

tested, 1.25×10^{-2} M, inhibited oxygen uptake with glycolic acid as substrate only 4 to 16 per cent. Oxidation of ascorbic acid was inhibited 24 per cent by 1.25×10^{-2} M 2,4,5-T. These authors conclude that their data "do not support the idea that herbicides work by stimulating respiratory enzymes, nor do they explain the differential effect of certain herbicides on mono- and dicotyledonous plants."

Bonner and Bandurski (15) have suggested that auxins (which would include 2,4-D and 2,4,5-T) may affect respiration principally through a phosphate transfer system. Loustalot et al. (48) treated bean plants with 0.1 per cent aqueous sodium 2,4-D and analysed them for inorganic phosphate. They suggested that 2,4-D might inhibit or poison the enzyme or enzyme system responsible for the synthesis of high-energy phosphate bonds. Such an event would lead to increased catabolic activity which could prevent growth or anabolism (45). Thus the possibility that herbicides of the 2,4-D type affect plants through action on the phosphorylation mechanism seems like as good a working hypothesis as any available.

In experiments of this type the possibility of contamination of the 2,4-D with dichlorophenol (DCP) should not be overlooked. DCP has been shown to be present in 2,4-D in sufficiently high quantities to affect the results obtained with this herbicide (29). In addition, DCP is known to be a potent uncoupling agent (25). Of course 2,4-D, which is structurally similar to DCP, may also act in uncoupling

oxidation and phosphorylation (17), but such a conclusion should not be arrived at without some information on the DCP content of the test material. Even with pure 2,4-D the possibility exists that at least some of its effects are brought about only after it has been broken down in the plant, perhaps to DCP (77). The importance of dichlorophenol in studies of 2,4-D-plant relationships is evident. A more complete discussion of the literature pertaining to this chemical will be found in another section.

Other chemicals

Certain chemicals belonging to such varied general classes as herbicides, anti-auxins, and anti-metabolites have been shown to affect plant respiration. A few of these chemicals were tested on mitochondria in this study. The literature pertaining to the chemicals used is reviewed below.

Sodium chlorate. This herbicide has been widely used for many years as a soil sterilant, but its exact mode of action is still unknown (2). Latshaw and Zahnly (43) concluded that part of the killing effect was due to a depletion of food reserves, and Neller (57) found that sodium chlorate decreased catalase activity. A temporary increase in the respiration rate of treated plants has also been noted (2). The high toxicity of sodium chlorate may be due to the high oxidizing ability of the chlorate ion, or to the presence of the pentavalent chlorine (2). In either case changes in

respiration might be expected.

Trichloroacetic acid (TCA). Generally used in the form of the sodium salt, this chemical was found by Kelly and Avery (38) to have little effect on the respiration of Avena coleoptiles although a slight stimulation was noted at concentrations of 0.01 to 0.1 mg per liter. Miller and Burris (50) observed no effect of TCA at concentrations from 10^{-4}M to 1.25×10^{-2}M on the oxidation of glycolic or ascorbic acids by cell-free enzymes from barley plants. Rebstock et al. (63), however, found that oxygen uptake was greater in both the shoots and roots of wheat seedlings grown in soil that had been treated with TCA. This increased respiration was evident 10 to 20 days after treatment, but no stimulation was observed after three days. TCA is often used in chemical analysis as a protein precipitant so it may affect plant metabolism by protein precipitation (2). Since enzymes are primarily protein in nature, such an effect would soon lead to profound changes in the respiratory process.

2,2,Dichloropropionic acid (DCPA). This relatively new herbicide, marketed under the trade-name Dalapon, has been shown to bring about malformation of new growth of grasses (21). In general it appears to affect plants in a similar manner to TCA, except that DCPA is translocated to growing points to a greater extent. No information on the effect of DCPA on plant respiration has yet appeared. In this connection the possibility of DCPA being hydrolysed to pyruvic acid is of interest. Such hydrolysis has been shown to take

place readily in water solution (21). If DCPA is also subject to hydrolytic breakdown in plants, respiratory changes might be expected as a result of increased pyruvate concentration.

Herbicidal oils. The early literature on the effect of oils upon plant respiration has been reviewed by Ahlgren et al. (2). Oils were found to increase the rate of respiration of some plants and to decrease that of others. Greene (31) showed that poorly refined oils generally gave a greater increase in respiration than more highly refined oils. Johnson and Hoskins (36) found that the toxicity of spray oils to bean plants was correlated with their acid content. Neutral, non-peroxidic oils had only a small effect on oxygen absorption, whereas oxidized, acidic oils reduced oxygen uptake by leaf fragments by as much as 30 per cent. This inhibition was not reversed by succinic, fumaric, malic or pyruvic acids. The authors concluded that oxidized oils do not specifically inhibit the succinic dehydrogenase system. They also showed that these oils had no effect on the in vitro activity of the cytochrome oxidase system of bean leaf brie.

Maleic hydrazide. This compound inhibits growth, apparently by lowering auxin effectiveness in plants (45). Andreae and Andreae (3) showed that maleic hydrazide stimulated IAA oxidation and suggested that this was the manner in which it controls growth. There was no effect on oxygen up-

take of tomato stem, crown-gall tumor tissue, or Agrobacterium tumefaciens treated with 100 to 1000 ppm maleic hydrazide (39). Similarly, Naylor and Davis (56) found that concentrations of maleic hydrazide from 500 to 4000 ppm had little effect on the respiration of root tips at pH 6.0. However, a marked inhibition was observed at pH 4.0. Pea and corn root tips were least affected, and oats, wheat, and barley most affected. These authors (56) concluded that it is possible that maleic hydrazide influences growth by inhibiting respiration. They speculated that a dehydrogenase was prevented from functioning normally.

2,3,5-Triiodobenzoic acid (TIBA). Few, if any, papers describing the effect of TIBA on plant respiration have appeared. Observations such as those described below indicate that this chemical might bring about changes in respiration similar to those brought about by auxin. Galston (26) found that TIBA suppressed the activity of auxin in the Avena curvature test, and inhibited both internode elongation and apical dominance in soybean. High concentrations of TIBA (50 ppm) were also strongly inhibitory in the Avena straight growth test (74). At 0.5 ppm, however, TIBA had no effect on Avena when used alone, but in the presence of 1 ppm IAA it increased growth about 30 per cent over the auxin alone. In mixtures with 2,4-D, TIBA was found to be either additive or antagonistic depending upon the concentration used (1).

Coumarin. This chemical and other unsaturated lactones

have long been known to inhibit growth (45). Audus and Quastel (4) showed that while coumarin is active only at concentrations of the order of 10 ppm, it has formative effects similar to those of 2,4-D. They concluded that both coumarin and 2,4-D probably affect growth by forming "loose combinations or easily dissociated compound with enzymes or metabolites in the plant cell." Since no growth inhibition by coumarin occurs in the presence of sulfhydryl protecting agents, sulfhydryl groups may be involved (45). A change in respiration might be expected as part of the effect.

2,4-Dinitrophenol (DNP). As a group, the dinitrophenols have been classified as contact herbicides by Ahlgren et al. (2). Low concentrations of DNP stimulate respiration, and high concentrations strongly inhibit it (2, 58). A concentration of 5 ppm has been shown to inhibit growth 88 per cent while increasing respiration 38 per cent (15). Beevers (7) found that concentrations of DNP near 10^{-5}M stimulated respiration in a variety of tissues. In storage organs such as carrot a greater stimulation was observed than in rapidly dividing tissue of corn root tips. These results provide further support for the idea that DNP acts by uncoupling phosphorylation from oxidation (15). In the semi-dormant carrot in which the capacity of the phosphorylating system limits rate of respiration (few phosphate acceptor-sites are available) a large stimulation is obtained with DNP. In the meristematic tissues of the corn root tips where high energy bonds are being utilized rapidly in growth, the

respiratory rate is not limited by lack of phosphate acceptors, and hence little stimulation of respiration is brought about by DNP. Since growth in this tissue is dependant on high-energy bonds, and since DNP inhibits the formation of ATP (16), growth is inhibited. In the presence of DNP, plants or plant systems are also unable to carry out such energy-requiring processes as salt-uptake, water-uptake, and enzyme synthesis (15).

2,4-Dichlorophenol (DCP). Some of the responses attributed to 2,4-D, such as enhancement of the activity of the enzyme indoleacetic acid oxidase, have been shown to be caused by contamination with DCP (29). A sample of 2,4-D active in stimulation of this enzyme had a total phenol content of 0.3 per cent (mole/mole). Inactive samples contained 0.02 per cent total phenol. Goldacre et al.(29) believed that DCP acted as an activator of IAA oxidase, because of a structural similarity to some natural cofactor, or as an electron carrier in the oxidative system. Lockhart (47) studied the role of DCP in the destruction of IAA by peroxidase. He concluded that DCP was effective in the peroxidase portion of IAA oxidase rather than on the physiological generation of peroxide. Other enzymes, including cytochrome oxidase of mung bean mitochondria, were treated with DCP by Goldacre and Galston (28). They found that 10^{-5} or 10^{-4} M DCP had no effect on oxygen uptake by these mitochondria in the presence of cytochrome (0.5 mg/ml), but slight inhibitions (12 per cent) were observed at 10^{-3} M. Catalase was 50 per

cent inhibited by 2×10^{-6} M DCP, and peroxidase and hemoglobin were unaffected by concentrations below 10^{-4} M.

French and Beevers (25) found that a concentration of 5×10^{-4} M DCP stimulated oxygen uptake of corn coleoptile segments. This stimulation was accompanied by growth inhibition, indicating that DCP acts as an uncoupling agent in a similar manner to DNP.

Effect of Herbicides and Related Chemicals on Mitochondrial Respiration

Although Millerd et al. (53) showed in 1951 that particles isolated from mung beans could carry out complete oxidation of pyruvic acid to carbon dioxide and water, very little has been published on the effect of auxins on this enzyme system. Bonner and Bandurski (15) and Leopold (45) cite the unpublished work of Price et al. They found that the rate of pyruvate oxidation in vitro was not influenced by added auxin nor by pretreatment of the living plant with IAA. These observations suggest that auxin may not affect respiration by acting on Krebs cycle enzymes, and have been used by Bonner and Bandurski (15) as indirect evidence that the action of auxin is concerned with the phosphate transfer mechanism.

In an unpublished Master's thesis, Dow (22) noted the effects of growth substances on the oxidative activity of mitochondria isolated from lupine seedlings. Among others,

he tested the chemicals IAA, 2,4-D, 2,4,5-T, and maleic hydrazide at concentrations of 10^{-2}, 10^{-3}, and 10^{-4} M. In general, he obtained only slight stimulation of oxygen uptake with the lowest concentrations of IAA, 2,4-D, and 2,4,5-T and inhibition at the highest concentration. Maleic hydrazide had very little effect. A more complete discussion of Dow's results will be made in connection with the results of the present study.

Brody (17) studied the effects of plant growth substances on the oxidative phosphorylation of rat liver mitochondria. He found that 2,4-D was a potent uncoupling agent. At 10^{-3} M, 2,4-D had little effect on respiration but the P:O ratio was depressed to 20 per cent of the control. In a phosphate deficient system, increased concentrations of 2,4-D resulted in increased respiratory rates and increased liberation of inorganic phosphate. This uncoupling effect was visible at concentrations as low as 5×10^{-5} M. These observations help substantiate the hypothesis of Bonner and Bandurski (15) discussed earlier, that the changes in respiration brought about by 2,4-D are due to the action of this herbicide on the phosphorylation system. Since no data on the DCP level of the 2,4-D used were given, the possibility remains that the uncoupling effect was brought about by DCP contamination rather than by the 2,4-D itself.

MATERIALS AND METHODS

Chemicals

Source of chemicals

Chemicals used in this study were obtained from the sources listed below.

Krebs cycle acids	Source
Sodium citrate	Eimer and Amend
d-l-Glutamic acid	Merck and Co.
-Ketoglutaric acid	General Biochemicals
l-Malic acid	Eastman Chemicals
Oxaloacetic acid	Organic Specialities
Sodium pyruvate	Schwarz Laboratories
Succinic acid	Mallinckrodt Chemicals

Cofactors	Source
Adenosine-5-phosphate (AMP)[a]	Pabst Laboratories
Adenosine diphosphate (ADP)[a]	Pabst Laboratories
Adenosine triphosphate (ATP)[a]	Schwarz Laboratories
Coenzyme A (Co A) 75%[b]	Pabst Laboratories
Cytochrome c (Cyt. c), 90-100%	Sigma Chemical Co.
Diphosphopyridine nucleotide (DPN)[a]	Schwarz Laboratories
Hexokinase[c]	Pabst Laboratories

Herbicides and related chemicals	Source
Sodium chlorate, 99%	Chipman Chemicals
Coumarin	Merck and Co.
2,4-Dichlorophenol (DCP)	Eastman Chemicals
2,4-Dichlorophenoxyacetic acid (2,4-D)	Eastman Chemicals
Sodium 2,4-dichlorophenoxyacetate 85% acid equivalent	Baker Chemical Co.
2,2-Dichloropropionic acid (DCPA) 99%	Dow Chemical Co.
2,4-Dinitrophenol (DNP)	Eastman Chemicals
Indoleacetic acid (IAA)	Eastman Chemicals
Herbicidal oil L-8764	Standard Oil Co., Ind.

Maleic hydrazide, sodium salt	U.S. Rubber Co.
2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)	Dow Chemical Co.
2,3,5-Triiodobenzoic acid (TIBA)	Eastman Chemicals
Sodium trichloroacetate (TCA), 90%	Dow Chemical Co.
Wetting agent (Triton B-1956)	Rohm and Haas

[a]Chromatographically pure

[b]Equivalent to 300 Lipmann units/gm

[c]Contained 28,000 Kunitz-McDonald units/gm at 30°C

Preparation of herbicides and related materials

All chemicals tested for effect on mitochondrial respiration were made up in water solution at eight times the final concentration desired in the reaction mixture. In each case a stock solution was prepared and a range of concentrations produced by serial dilution. Acids not readily soluble in water (2,4,5-T, IAA, TIBA) were dissolved by adding a few ml of 0.25 N sodium hydroxide. After solution was attained the excess alkali was neutralized to pH 7 with hydrochloric acid. Maleic hydrazide and 2,4-D were obtained in the form of the sodium salts and were thus readily soluble in cold water, as were the other compounds investigated (TCA, sodium chlorate, coumarin). The herbicidal oil tested (Standard L-8764) was emulsified by the addition of a wetting agent (Triton B-1956) or added without emulsification to the flask contents.

Experimental Methods

Preparation of active mitochondria

In general, the procedures of Millerd *et al.* (51) with mung beans, and Laties (42) with cauliflower buds, were followed in the isolation of active particles from soybean hypocotyls. Soybeans (*Glycine max* (L) Merr. var. Hawkeye) were surface sterilized for 10 minutes in 0.5 per cent sodium hypochlorite, and soaked in tap water for approximately 24 hours. The beans were then planted in sterilized sand in shallow enamel pans. They were grown in the dark in 26°C incubators for four days. At this time, when they were approximately 4 inches in height, the seedlings were harvested. The cotyledons and roots were removed and the hypocotyl was transferred to a cold beaker kept in ice water. Throughout the entire procedure ice baths were used to keep the plant material close to 0°C. Steps that had to be carried out at laboratory temperature, such as balancing the centrifuge tubes, were performed rapidly so that the temperature of the material did not rise appreciably.

From 80 to 100 g of tissue was used in each experiment. After weighing, the hypocotyls were cut into small pieces with scissors and divided into two lots of 40 to 50 g. Each lot was macerated for approximately 2 minutes in a chilled mortar with cold quartz sand and 25 ml of sucrose-phosphate solution. This solution contained 0.4 M sucrose and 0.1 M

phosphate (KH_2PO_4 + Na_2HPO_4) and had a pH of 7.1. After grinding, the pH of the brei was approximately 6.8. The brei was strained through cheesecloth to remove most of the sand and tissue debris, and centrifuged for 5 minutes at 500 times gravity in an International No. 2, refrigerated centrifuge. The residual suspension was decanted into four plastic tubes and recentrifuged at approximately 10,000 times gravity for 15 minutes in the high speed head of the centrifuge. The supernatant from this centrifugation was decanted off and discarded. The residue in each tube was suspended in 2 ml of a cold solution, 0.4 M in sucrose and 0.1 M in pH 6.9 phosphate, by means of a loose-fitting plastic pestle. The total volume was brought to 20 ml and this suspension recentrifuged in two tubes at approximately 10,000 times gravity for 15 minutes. The precipitate in each tube from this second high-speed centrifugation was suspended in 1.0 ml of sugar-phosphate solution and transferred to a glass homogenizing tube. The volume was adjusted to 8 or 15 ml and the suspension homogenized by means of a snug-fitting (peripheral clearance 0.05 to 0.075 mm) power-driven plastic pestle. Either 0.5 or 1.0 ml of this homogenate containing approximately 0.4 mg of nitrogen was used in each Warburg flask.

In most experiments all materials other than the enzyme preparation were placed in the flasks while the enzyme was being prepared. The enzyme was then added immediately

following the final homogenization. In a few cases, however, the homogenate was allowed to stand in an ice bath for periods up to 30 minutes before addition to the flasks. This delay had no appreciable effect on the oxidative activity of the particles.

Nitrogen determination

In each experiment duplicate samples of the homogenate of the same volume as used in the experiment (0.5 or 1.0 ml) were set aside for nitrogen determination. In most instances nitrogen was not determined at once, but the samples were frozen and determinations made for several experiments at a convenient time. Digestion was carried out in micro-Kjeldahl flasks in a digestion mixture of approximately 10 mg powdered selenium, 50 mg of a 3:1 $CuSO_4$ - K_2SO_4 mixture, and 2 ml concentrated sulfuric acid. Nitrogen was determined as ammonia released from the digestate by 30 per cent sodium hydroxide. The ammonia was steam distilled into 2 per cent boric acid containing methyl red-methylene blue indicator, and titrated with 0.01 N hydrochloric acid. Mitochondrial nitrogen varied between 0.3 and 0.6 mg per flask, but in most experiments was approximately 0.4 mg.

Respiratory measurements

Oxygen uptake was determined manometrically at 30°C by the method of Warburg as outlined by Umbreit et al. (75). All reagents were made up at eight times the final concentration in the reaction mixture, and neutralized to pH 7.

Usually each reagent was made up in a separate solution, but in certain experiments some of the cofactors were combined. In all cases the reaction mixture had a total volume of 2.0 ml and the fluid volume in the flask was 3.0 ml. Preliminary experiments indicated that there was little or no advantage to placing the enzyme in a side-arm and mixing it with the rest of the flask contents at zero time. The complete reaction mixture was therefore placed in the body of the flask before equilibration. The enzyme suspension was always added last, just before the flasks were attached to the manometers.

Carbon dioxide evolution was determined at the end of the experiments, mainly in the same two-side-arm flasks, in which oxygen uptake was measured (75). In this one-flask method, 0.3 ml of 20 per cent potassium hydroxide was placed in one double side-arm and 0.4 ml of 4 N hydrochloric acid in the other. At the end of the experiment the carbon dioxide absorbed by the alkali was released by tipping in the acid. The original carbon dioxide content of the potassium hydroxide used in these experiments was kept low by precipitation as barium carbonate, and was determined at the time of each experiment. This value was then subtracted from the amount of gas released from the alkali when the acid was tipped in.

In most experiments the manometers were read at 5 minute intervals for 30 minutes. In all cases gas exchanges

are net values (total minus endogenous) calculated from the 30 minute reading and expressed as microliters per mg nitrogen per hour ($Q_{O_2}(N)$).

Phosphate measurements

In experiments in which phosphorylation was measured, an initial phosphate concentration of 0.00625M was used in the reaction mixture. Inorganic phosphate was determined at zero time and at the end of 35 minutes. The difference in these two determinations was considered to represent the amount of inorganic phosphate esterified by the enzyme system. The zero determination was taken from one flask after equilibration, and the 35 minute determination from individual flasks in which oxygen uptake had been measured for 30 minutes.

In this procedure 0.5 ml of the reaction mixture was removed from a flask and added to 1.5 ml of 2 N hydrochloric acid in a test tube kept in an ice-bath. After similar aliquots had been taken from all flasks and added to a series of test tubes, the mitochondria in each sample were removed from suspension by high speed centrifugation (16). An 0.5 ml aliquot of the clear supernatant was placed in a colorimeter tube and the inorganic phosphate determined in a Klett colorimeter by the method of Fiske and Subbarow (23). A standard curve was set up, using dilutions of a solution made from oven-dried potassium acid phosphate. Light absorption was proportional to phosphate concentration over

a range from 0.25 to 2.0 micromoles (Figure 1). The amount of phosphate in the unknowns was determined by comparison with this standard curve. The number of micromoles thus determined was multiplied by sixteen (the dilution factor) to give the micromoles of phosphate in the Warburg flask at the time of measurment. The amount esterified was then determined by difference as outlined above, and expressed as micromoles per hour.

Phenol measurments

Phenol was determined colorimetrically by the method of Folin and Ciocalteau (24). Serial dilutions of 2,4-dichlorophenol were used to establish a standard curve and the quantity of phenol in the unknowns was determined by comparison with this curve (Figure 2).

Measurements of hypocotyl-segment respiration

In some experiments, the effects of certain herbicides on the respiration of hypocotyl segments were investigated for comparison with effects on isolated mitochondria. Soybeans were grown in the same manner and harvested at the same time as those used as a source of mitochondria. After harvest, two or three hypocotyls were blotted dry, weighed, cut into segments approximately 5 mm long, and placed in Warburg flasks. The flasks were kept in ice until all were ready to attach to the manometers. The hypocotyl segments were floated in 2 ml of water plus herbicide, and the total fluid content of each flask was brought to 3 ml as in other

Figure 1. Standard curve showing relationship between phosphate content and optional density.

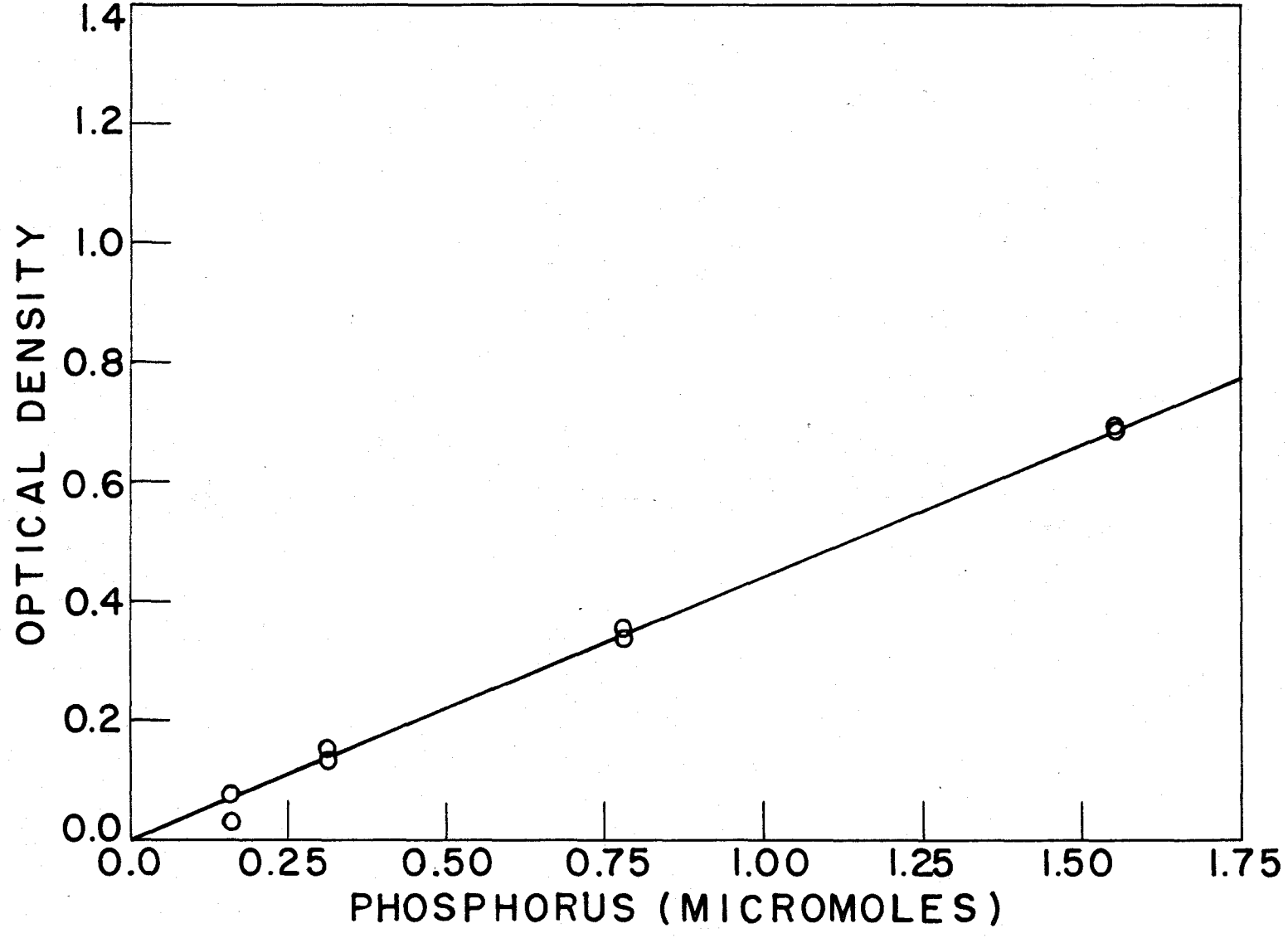
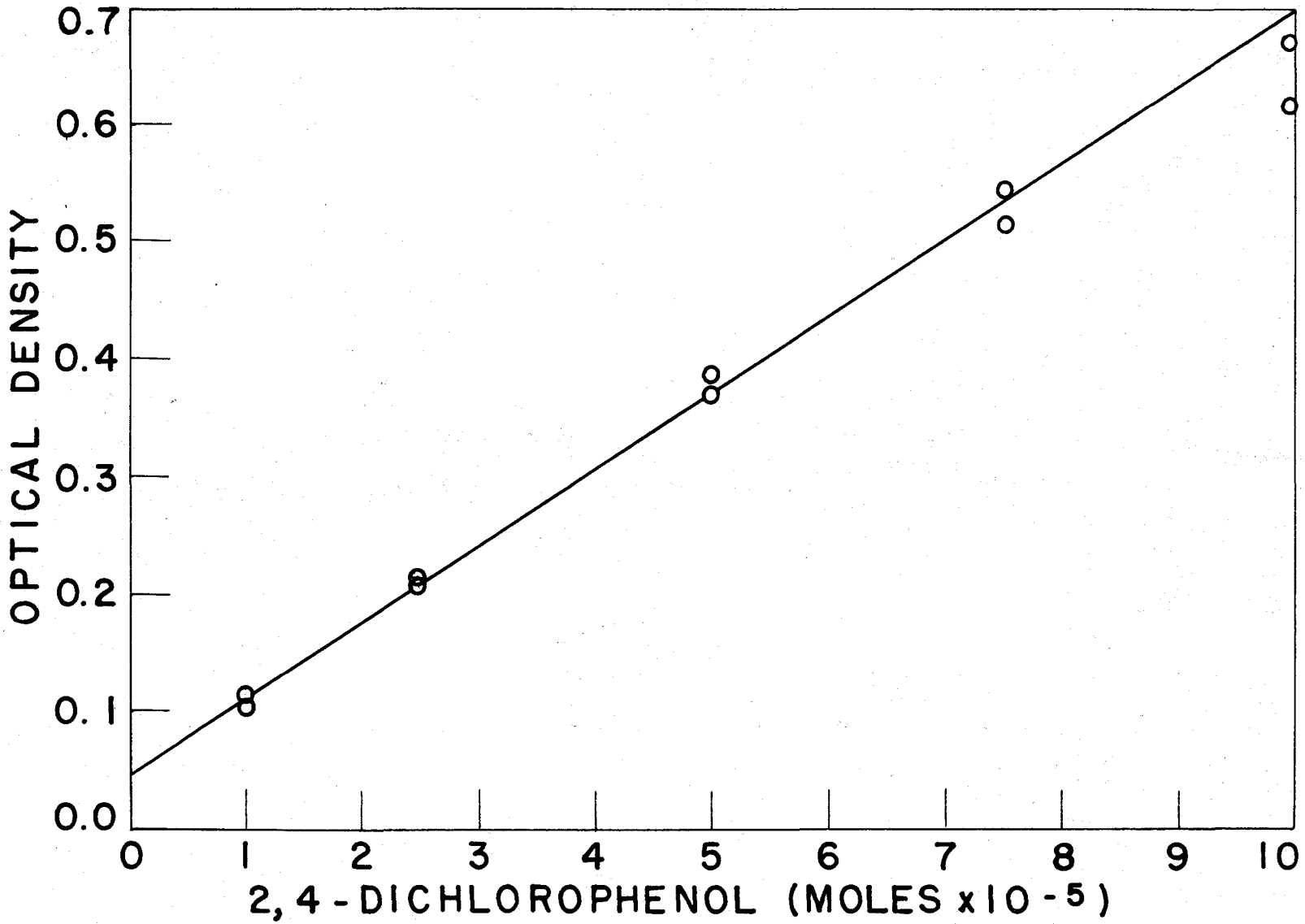

OPTICAL DENSITY
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.25
0.50
0.75
1.00
1.25
1.50
1.75
PHOSPHORUS (MICROMOLES)

Figure 2. Standard curve showing relationship between dichlorophenol concentration and optical density.


OPTICAL DENSITY
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
1
2
3
4
5
6
7
8
9
10
2, 4 - DICHLOROPHENOL (MOLES x 10 - 5)

experiments. The technique of oxygen and carbon dioxide measurment was the same as outlined for mitochondrial respiration determination. Gas exchange was expressed as microliters per hour per gram fresh weight.

Pretreatment of soybeans

The effect of spraying etiolated soybeans with 2,4-D upon the subsequent activity of isolated mitochondria was tested in several experiments. Various concentrations of this herbicide were used at different times prior to harvest. Spraying was done with a DeVilbiss atomizer and enough spray applied to wet thoroughly all above-ground parts of the plant. One-half a pan of plants was sprayed in each experiment, the other half acting as control. The control plants were protected by a cardboard screen during spraying, and this operation was carried out as rapidly as possible in dim light.

Cytological studies

Possible changes in the size and/or shape of soybean particles during the isolation procedure were investigated. Sections from etiolated soybean hypocotyls were fixed in Zirkle's modification of Erliki's fluid (65). These sections were dehydrated, embedded, sectioned, and stained following the recommendations of Sass (65). The dark-stained, mostly spherical particles may be seen in cells of the cortex in Figure 3. The particles in the final homogenate were also observed and are shown under comparable magnification in

Figure 4. Apparently the isolation procedure had little visible effect on the morphology of the particles.

Figure 3. Appearance of mitochondria in cortical cells of etiolated soybean hypocotls.

Figure 4. Appearance of soybean mitochondria after isolation in sucrose-phosphate solution.

EXPERIMENTAL RESULTS

Characterization of Soybean Mitochondria

Relation of activity to enzyme concentration

Preliminary experiments indicated that soybean mitochondria isolated as outlined in the methods section were capable of oxidizing certain Krebs cycle intermediates. The rate of oxygen uptake appeared to be proportional to the concentration of the particulate suspension in each flask. Since such a relationship, at least within a given lot of enzyme, is essential in a study such as was planned, experiments were set up to investigate this proportionality.

Of the various Krebs cycle intermediates tested, succinate and pyruvate were chosen as oxidative substrates in most experiments. These two have been widely used by other workers and were found in this study to be oxidized more rapidly than any other by soybean mitochondria. These substrates were used at concentrations of 2×10^{-2} M throughout all experiments. A low concentration of l-malate (1.7×10^{-3} M) was added to pyruvate to serve as a "sparker" (see later experiments on effect of this addition).

The results of experiments to test the proportionality between enzyme concentration, expressed as mg nitrogen, and rate of oxidation with succinate and pyruvate as substrates are presented in Figures 5 and 6. In each experiment the highest concentration of enzyme was diluted with sucrose-

Figure 5. Relation between succinate oxidation and enzyme concentration.

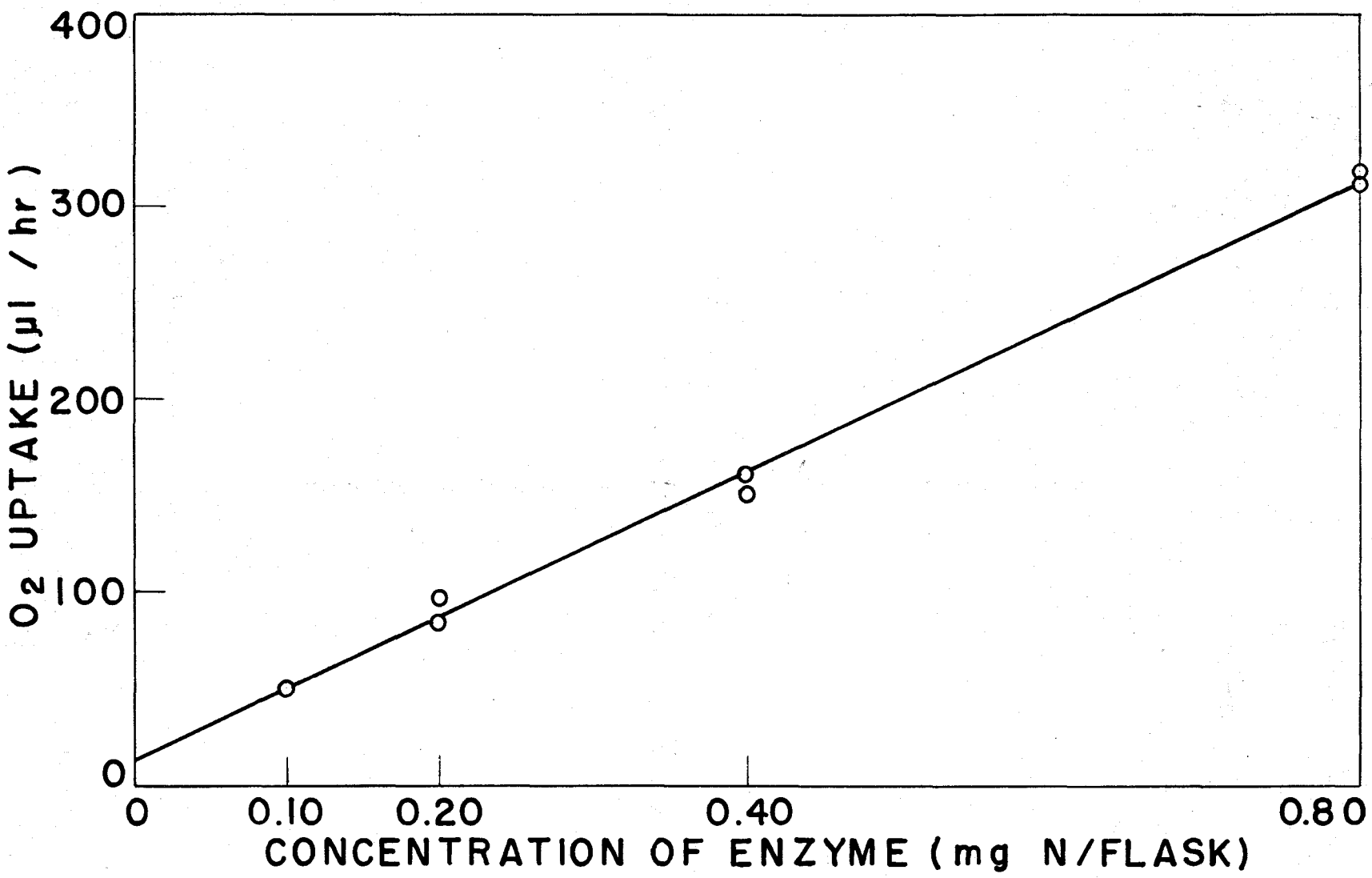
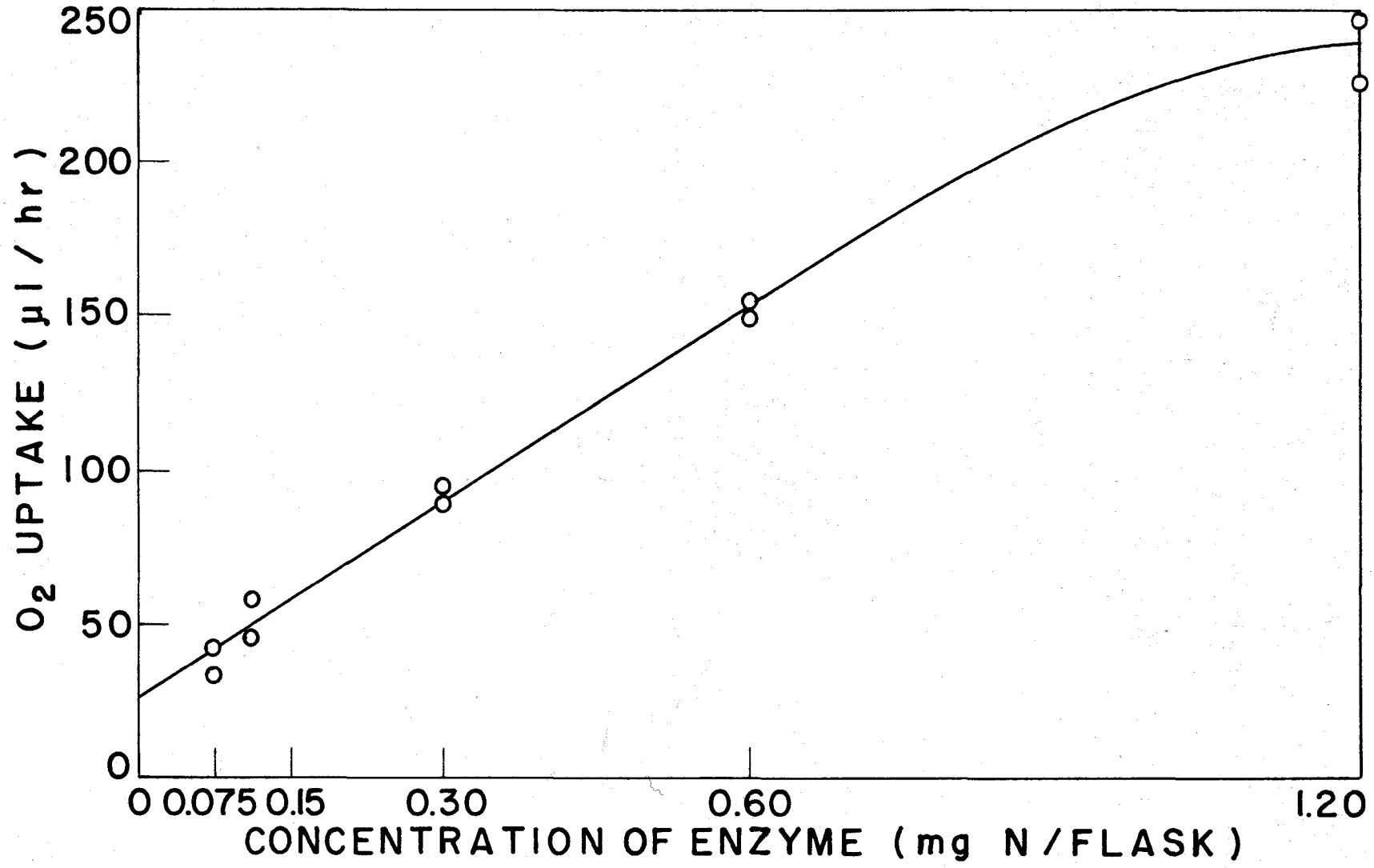

400
300
200
100
0
O_2 UPTAKE (µl / hr)
0
0.10
0.20
0.40
0.80
CONCENTRATION OF ENZYME (mg N/FLASK)

Figure 6. Relation between pyruvate oxidation and enzyme concentration.


250
200
150
100
50
0
O_2 UPTAKE (µl / hr)
0 0.075 0.15 0.30 0.60 1.20
CONCENTRATION OF ENZYME (mg N / FLASK)

phosphate solution to give the other concentrations shown. With succinate as substrate (Figure 5), a good proportionality is shown over the range of enzyme concentrations tested. With pyruvate as substrate (Figure 6) response was also linear over this concentration range, but there was some dropping off at higher enzyme levels. Enzyme concentrations used throughout this study were, however, in the range of 0.3 to 0.6 mg nitrogen per flask, which is on the straight-line portion of both curves.

Decline in rate of oxidation with time

The oxidative activity of soybean particles in the reaction mixture was found to diminish with time. The results of a typical experiment in which oxygen uptake was recorded for a period of one hour are shown in Figure 7. It may be seen that pyruvate oxidation was proportional to time for approximately 20 minutes, but succinate and ketoglutarate oxidation began to fall off even sooner. There was little endogenous oxygen uptake after 30 minutes. A similar decline in activity has been observed with particles isolated from Avena (72) and from cauliflower (41). No attempt was made to arrest this diminishing activity in the present study.

Relative activity of soybean and mung bean mitochondria

Much of the earlier work with plant mitochondria was carried out with particles isolated from mung beans, Phaseolus aureus (52). As the activity of this material seems to have been well characterized, a few experiments

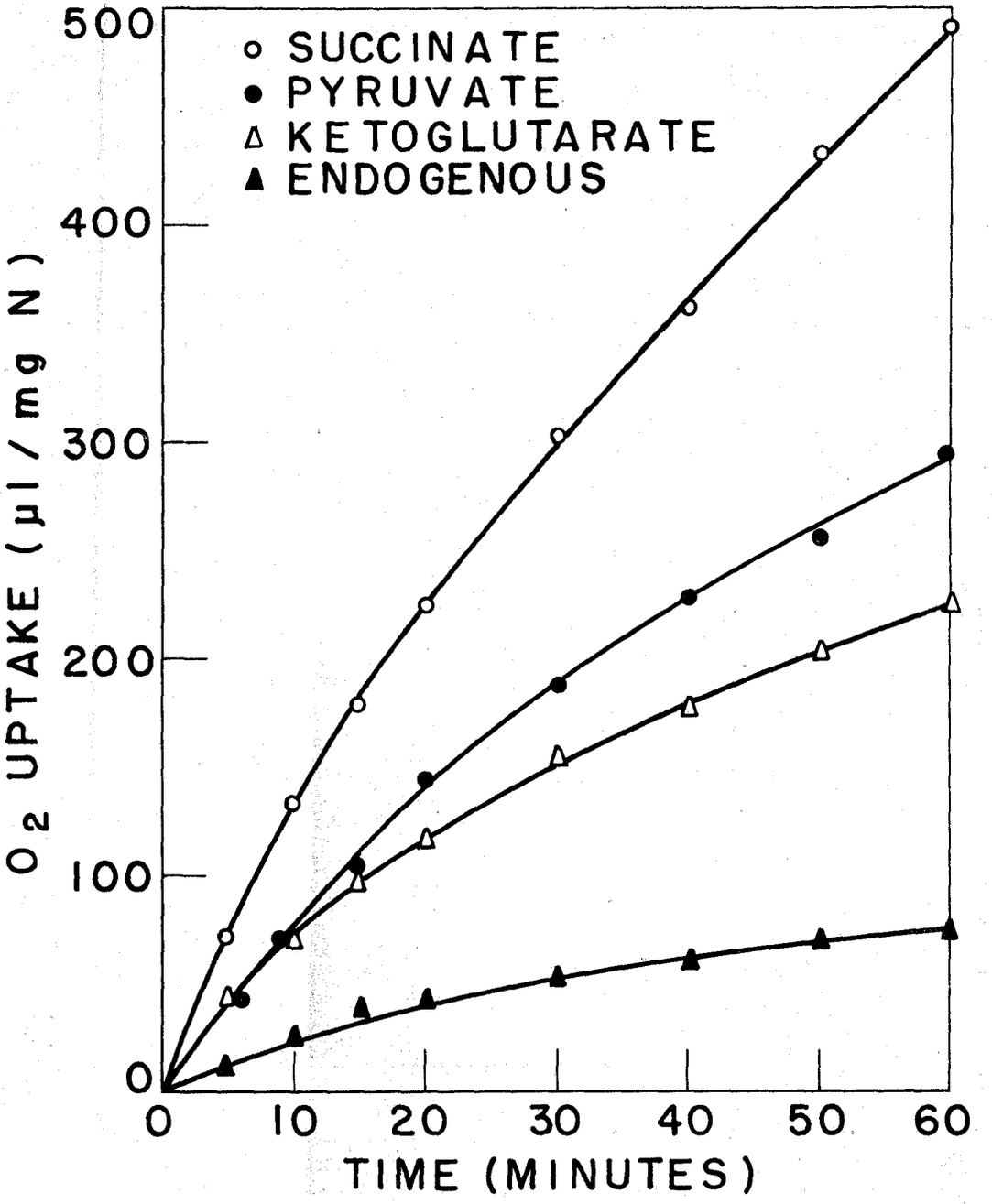


Figure 7. Decline in activity of soybean mitochondria with time (in reaction mixture at 30°C).

were conducted using mung bean mitochondria to compare the results obtainable under our laboratory conditions with those obtained by others. The usual concentration of substrate (2×10^{-2}M) was used, and malate (1.7×10^{-3}) was added to the flasks containing pyruvate. ATP (5×10^{-4}M) and $MgSO_4$ (10^{-3}M) were added to all flasks except where otherwise noted. The results of a representative experiment in which both soybean and mung bean mitochondria were used are presented in Table 1.

These data show close agreement between our results and those of Millerd *et al.* (53) with mung bean mitochondria. One exception was the strong stimulation of pyruvate oxidation obtained by these investigators upon the addition of magnesium ions. Although magnesium did not stimulate oxygen uptake by our mung bean mitochondria, it was effective when added to soybean preparations. In general, however, the oxidative activity of particles prepared from soybeans seems to be similar to that of mung bean mitochondria.

Ability of soybean mitochondria to oxidize Krebs intermediates

The data in Table 2 show that while soybean mitochondria have the ability to utilize all Krebs cycle intermediates tested as substrate, some are oxidized more rapidly than others. The largest oxygen uptake was observed with succinate as substrate, followed by pyruvate (plus a small amount of malate), citrate, and ketoglutarate. Without the

Table 1. Relative activities of soybean and mung bean mitochondria.[a]

Substrate	Reaction mixture	$Q_{O_2}(N)$	
		Soybeans	Mung beans
Succinate	Complete	570	467 (202)[b]
Ketoglutarate	Complete	202	225 (220)
Pyruvate	Complete	156	165 (105)
Pyruvate	Complete less ATP and $MgSO_4$	70	68 (60)
Pyruvate	Complete less $MgSO_4$	107	101 (97)
Pyruvate	Complete less ATP	84	70 (135)

[a]Complete reaction mixture contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M; malate, 0.0017 M, added with pyruvate.

[b]Data in parentheses from Millerd et al. (53).

addition of malate, pyruvate oxidation was very slow. The quantity of malate used as sparker (1.7×10^{-3} M) was sufficient to bring about a small amount of oxidation by itself. Glutamate also was utilized, indicating the ability of isolated soybean mitochondria to oxidize at least this one amino acid. There was only slight oxidation of fumarate and oxaloacetate although malate (2×10^{-2} M) was utilized to a greater extent.

The r.q. data of Table 2 are important in that they provide a clue to the type of reactions. Values near theoretical indicate that oxidation was proceeding essentially to carbon dioxide and water. Lower figures, as those obtained for succinate oxidation, indicate that the substrate was being oxidized rapidly but that the product of this oxidation (fumarate) was probably changed slowly). Average r.q. values for pyruvate, ketoglutarate, and succinate obtained from many experiments in this study were 1.3, 1.2, and 0.3, respectively. These are similar to those obtained by Millerd et al. (53) for pyruvate metabolism by mung bean mitochondria, and by Hackett and Simon (32) for ketoglutarate and succinate metabolism by mitochondria from Arum maculatum.

Effect of certain changes in procedure on oxidative activity

Composition of grinding and suspending media. The tonicity, phosphate content, and pH of the solutions in which plant mitochondria are placed have been shown to have an influence on the oxidative activity of these particles (42,

Table 2. Ability of soybean mitochondria to oxidize various Krebs cycle intermediates.[a]

Substrate	$Q_{O_2}(N)$	$Q_{CO_2}(N)$	R.q.	
			Theoretical[b]	Observed
Pyruvate	7	-	-	-
Malate (0.0017 M)	25	-	-	-
Pyruvate[c]	168	200	1.20	1.2
Citrate	130	205	1.33	1.6
Ketoglutarate	123	169	1.25	1.4
Glutamate	60	70	1.11	1.2
Succinate	319	96	1.14	0.3
Fumarate	13	11	-	-
Malate	65	75	1.33	1.2
Oxaloacetate	17	43	-	-

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M.

[b]Theoretical values based on complete oxidation to carbon dioxide and water.

[c]Malate, 0.0017 M, added.

50, 51, 53). The results of experiments to test the effects of such changes on soybean mitochondria are presented in Table 3. All figures are averages of two complete experiments In the first experiments of this type (A, Table 3), soybean hypocotyls were ground in the mortar, either with a solution of 0.4 M sucrose and 0.05 M KCl, or with a solution of 0.4 M sucrose and 0.05 M tris. buffer. The pH of the brei obtained when no buffer was added was considerably lower than that which contained tris. buffer. Both homogenates were spun at low speed, then at high speed. Part of each pellet centrifuged out in the high-speed head was resuspended in an aliquot of the original sucrose-KCl solution, and part in an 0.4 M sucrose-0.05 M phosphate solution. After recentrifugation and resuspension in the same solution as in the previous step, each of the four homogenates thus obtained was tested for ability to oxidize succinate, ketoglutarate, and pyruvate.

With succinate as substrate, oxygen uptake was not affected by the addition of tris. buffer to the grinding medium. A large increase was, however, obtained with both ketoglutarate and pyruvate. The addition of phosphate to the washing and suspending medium increased oxidation of succinate and ketoglutarate regardless of the grinding medium but had no effect on pyruvate. With all substrates the greatest oxygen uptake was obtained when the grinding medium was buffered and phosphate was added to the suspending medium. These results led to experiments in which phosphate

Table 3. Effects of composition of grinding and suspending medium on oxygen uptake by soybean mitochondria.[a]

Substrate	Grinding medium	Total molarity	pH after grinding	Washing and suspending medium	Q_{O_2} (N)
(A)					
Succinate	S + KCl	0.45	6.4	S + KCl	218
Succinate	S + KCl	0.45	6.4	S + PO_4	352
Succinate	S + tris.	0.45	6.9	S + KCl	221
Succinate	S + tris.	0.45	6.9	S + PO_4	438
Keto-glutarate	S + KCl	0.45	6.4	S + KCl	0
Keto-glutarate	S + KCl	0.45	6.4	S + PO_4	42
Keto-glutarate	S + tris.	0.45	6.9	S + KCl	114
Keto-glutarate	S + tris.	0.45	6.9	S + PO_4	175
Pyruvate	S + KCl	0.45	6.4	S + KCl	23
Pyruvate	S + tris.	0.45	6.9	S + KCl	206
Pyruvate	S + tris.	0.45	6.9	S + PO_4	206
(B)					
Succinate	S + PO_4	0.45	6.9	S + PO_4	570
Succinate	S + PO_4	0.45	6.9	PO_4	208
Succinate	PO_4	0.10	6.9	S + PO_4	113
Succinate	PO_4	0.10	6.9	PO_4	62
Keto-glutarate	S + PO_4	0.45	6.9	S + PO_4	202
Keto-glutarate	S + PO_4	0.45	6.9	PO_4	25

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose (= S), 0.2 M; phosphate, 0.05 M; tris, 0.05 M. Malate, 0.0017 M, added with pyruvate. Grinding media contained sucrose (= S), 0.4 M; phosphate, 0.05 M; or 0.1 M; tris, 0.05 M; KCl, 0.05 M.

Table 3. (Continued)

Substrate	Grinding medium	Total molarity	pH after grinding	Washing and suspending medium	Q_{O_2} (N)
Keto-glutarate	PO_4	0.10	6.9	S + PO_4	43
Keto-glutarate	PO_4	0.10	6.9	PO_4	0
Pyruvate	S + PO_4	0.45	6.9	S + PO_4	228
Pyruvate	S + PO_4	0.45	6.9	PO_4	13
Pyruvate	PO_4	0.10	6.9	S + PO_4	30
Pyruvate	PO_4	0.10	6.9	PO_4	0

was used in the grinding medium with and without sucrose, as well as in the suspending solution. Data from experiments of this type are presented in the second part of Table 3.

With all substrates, oxygen uptake was greatly reduced when either sucrose or phosphate was omitted from either the grinding or suspending solution. No ketoglutarate or pyruvate oxidation occurred when sucrose was omitted from both grinding and suspending media, although succinate was oxidized to a slight extent. Particles prepared by grinding in phosphate alone (0.1 M) were capable of some oxidation if sucrose was added to the suspending medium, but little or none if suspended in phosphate alone.

These experiments show that soybean particles isolated, washed, and suspended in a sucrose-phosphate solution buffered near pH 7 had greater activity than those treated in any other manner tested. These results are similar to those obtained by Millerd (51) with mung bean mitochondria, but differ from those of Laties (42) with cauliflower. Except where otherwise noted, such hypertonic sucrose-phosphate solutions were used throughout the present study.

Washing. As mentioned earlier, particles were washed by suspension in sucrose-phosphate solution, followed by high-speed centrifugation. Since endogenous oxygen uptake was not entirely eliminated by one washing, experiments were designed to study the effects of a series of washings on both endogenous oxidation and oxidation of certain substrates.

A portion of the particles from the first high-speed spin was resuspended in sucrose-phosphate solution and tested for oxidative activity. These were considered to have zero washing. The rest of the particles were washed as outlined earlier. After each washing an aliquot was tested for activity. The results of a representative experiment are presented in Table 4.

Table 4. Effect of washing on oxygen uptake by soybean mitochondria.[a]

Substrate	Q_{O_2}(N) Number of washings			
	0	1	2	3
Succinate	269	591	542	540
Pyruvate	35	98	68	50
None	102	48	35	27

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M. Malate, 0.0017 M, added with pyruvate.

Oxygen uptake with pyruvate as substrate was lower than average in this experiment. However, the relative activities after successive washings were typical of other experiments in which pyruvate was used. Each washing decreased endo-

genous oxygen uptake, but each washing after the first also decreased oxygen uptake in the presence of added substrate. The increased oxidation of both pyruvate and succinate by washed particles was probably brought about because of the removal of some inhibitor or inhibitors by the washing procedure. Additional washings had little effect on the oxidation of succinate but decreased pyruvate oxidation considerably. One washing was therefore used routinely throughout this study.

pH of reaction mixture. The effect of varying the pH of the reaction mixture on oxygen uptake was investigated with succinate, pyruvate, and ketoglutarate as substrates. In these experiments, 0.5 ml of the mitochondrial suspension (pH 6.9) was added to each flask. The pH was then changed to various levels by the addition of 0.5 ml of phosphate solution (KH_2PO_4 - Na_2HPO_4) buffered at different hydrogen ion concentrations. The pH of the flask contents was determined at the end of the experiment. The relative oxidative activity of mitochondria at each of a series of pH values is presented in Figure 8. With all three substrates the optimum extended over a range from about pH 6.5 to pH 7.1. Based on these results, the reaction mixtures in other experiments of this study were buffered at pH 6.9. This is a little lower than that used by most other investigators. Millerd *et al.* (53, 54) worked at a pH of 7.1, Laties (42) and Tager (72) at 7.3. Soybean mitochondria appeared to be about as active

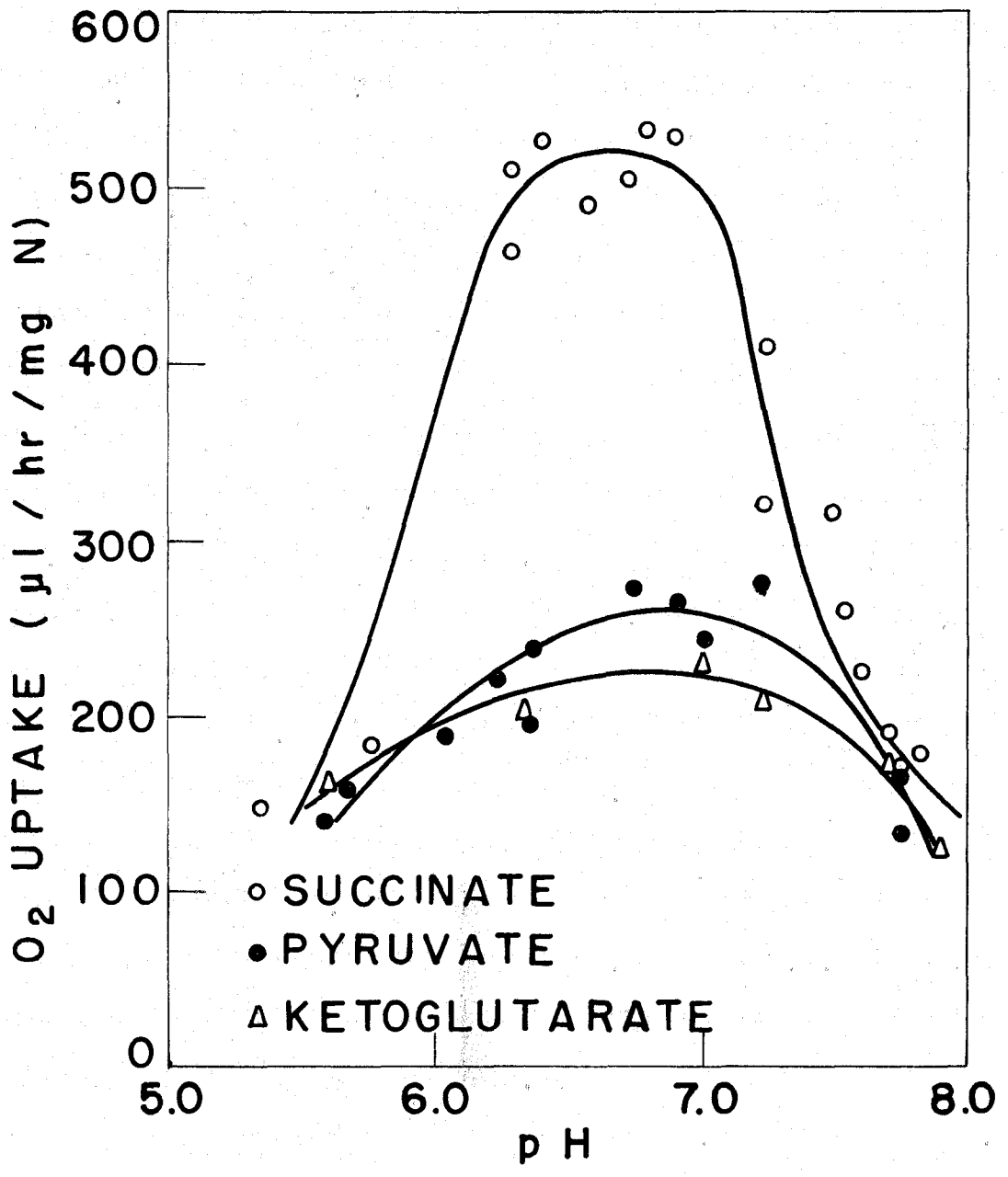


Figure 8. Effects of pH of reaction mixture on the oxidative activity of soybean mitochondria.

at pH 7.1 as at 6.9, but showed reduced activity, especially toward succinate, at pH 7.3. Whether this sensitivity to relatively high pH values is a distinct property of soybean mitochondria, or was due to something in the isolation procedure was not determined.

Addition of cofactors. ATP and $MgSO_4$ have already been shown to increase the oxidative activity of soybean mitochondria (Table 1). Additional experiments were carried out to test the effects of these and other cofactors. Data from representative experiments showing the effects of ATP, $MgSO_4$, and cytochrome c on oxidation of succinate, pyruvate, and ketoglutarate are presented in Table 5. The values for succinate and ketoglutarate are from one experiment, while those for pyruvate are from a different but comparable one. With all three substrates, oxygen uptake was increased by the addition of either $MgSO_4$ or ATP and still further increased if both were added. This increase was approximately additive with all three substrates. Pyruvate and ketoglutarate oxidations were stimulated more by ATP than by magnesium ions, and succinate oxidation showed the reverse. Pyruvate and succinate oxidation was further increased by the addition of cytochrome c. This cofactor had little effect on oxygen uptake in the presence of ketoglutarate.

The effects of the cofactors DPN and coenzyme A in mixtures containing magnesium ions and ATP were also investigated. Results of experiments of this type are summarized

Table 5. Effects of ATP, $MgSO_4$, and cytochrome c on oxygen uptake by soybean mitochondria.[a]

Substrate	Q_{O_2} (N)				
	Cofactors added				
	0	Mg	ATP	Mg+ATP	Mg+ATP+cyt. c
Succinate	200	397	319	570	765
Ketoglutarate	107	141	174	202	195
Pyruvate	70	84	107	156	171

[a]All flasks contained substrate, 0.02 M; sucrose, 0.2 M; phosphate, 0.05 M. Added were ATP, 0.0005 M; $MgSO_4$, 0.001 M; cytochrome c, 0.000028 M. Malate, 0.0017 M, added with pyruvate.

Table 6. Effects of DPN, coenzyme A, and cytochrome c on oxygen uptake by soybean mitochondria.[a]

Substrate	Q_{O_2} (N)				
	Cofactors added				
	0	DPN	Co A	DPN+Co A	DPN+Co A+cyt. c
Succinate	505	525	585	605	910
Ketoglutarate	122	171	195	224	249
Pyruvate	167	181	161	176	267

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose 0.2 M; phosphate, 0.05 M. Added were DPN, 0.00033 M; coenzyme A, 0.00067 M; cytochrome c, 0.000028 M. Malate, 0.0017 M, added with pyruvate.

in Table 6. Figures for oxygen uptake with pyruvate and ketoglutarate as substrate are from the same experiment; those for succinate from a separate but comparable experiment. DPN and Co A alone had little effect on either succinate or pyruvate oxidation, but in conjunction with cytochrome c they increased oxygen uptake markedly in the presence of both substrates. In the presence of ketoglutarate, both DPN and Co A stimulated oxygen uptake and cytochrome c had little additional effect.

In general, the data of Tables 5 and 6 show that oxidation of soybean mitochondria was stimulated by the addition of ATP, $MgSO_4$, DPN, Co A, and cytochrome c. Important exceptions appear to be the relative insensitivity of the ketoglutarate system to cytochrome c, and the pyruvate system to DPN and Co A.

Although the importance of added DPN, Co A and cytochrome c was recognized, only ATP and $MgSO_4$ were added routinely throughout this study. The greatest effect of cytochrome c was upon the succinoxidase system, and it was felt that succinate oxidation was already rapid enough that further increase was unnecessary.

In studies on phosphorylation it was desirable to replace ATP with AMP as a source of adenylate. Experiments were therefore set up to verify the equivalence of AMP, ADP, and ATP in stimulating oxygen uptake by soybean mitochondria. The results of a typical experiment of this type are shown in Table 7. It may be seen that the oxidation of succinate,

pyruvate, and ketoglutarate was stimulated to approximately the same extent by the addition of each of these adenylates.

Table 7. Equivalence of ATP, ADP, and AMP in the stimulation of oxygen uptake by soybean mitochondria.[a]

Substrate	Q_{O_2} (N)			
	Adenylate added			
	0	ATP	ADP	AMP
Succinate	308	445	438	433
Ketoglutarate	141	208	213	220
Pyruvate	170	220	217	213

[a]All flasks contained $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M. Malate, 0.0017 M, added with pyruvate.

Phosphorylation ability

Mitochondria isolated from other plants have been shown to utilize part of the energy of respiration in the formation of high-energy phosphate bonds (16, 42, 51, 53). Experiments were set up, therefore, to investigate the disappearance of inorganic phosphorous from the reaction mixture during the oxidation of certain substrates by soybean mitochondria. It was assumed that each micromole of inorganic phosphate that disappeared was attached through a high-energy bond to a micromole of adenylate, as has been shown with mung bean

mitochondria (16, 51). In other words, disappearance of inorganic phosphate was used as a measure of phosphorous esterification or of the phosphorylation ability of soybean mitochondria.

Particles were prepared in the usual manner except that a lower concentration of phosphate was used in the reaction mixture (6.25×10^{-3} M instead of 0.05 M). Other details of the procedure have been outlined in the methods section. AMP and glucose were added to all flasks except where otherwise noted. AMP was added as an acceptor for phosphate, as suggested by Bonner and Millerd (16), and glucose was added to ensure the trapping of high-energy phosphate as glucose-6-phosphate. This trapping reaction is mediated by the enzyme hexokinase, which has been shown to be associated with the mitochondria of potato tubers (64) and of mung beans (16). If it is similarly associated with soybean mitochondria in sufficient quantity to transfer all high-energy phosphate formed to glucose-6-phosphate, no increase in the rate of disappearance of inorganic phosphate would be expected from the addition of exogenous hexokinase. That such was not the case is shown in Table 8, which also shows the effects of added sodium fluoride on oxygen uptake and phosphate disappearance.

Some phosphorylation was evident in the presence of each of the three substrates tested when neither hexokinase nor fluoride was added. P:O ratios of 0.4, 0.8, and 0.9

Table 8. Effects of added hexokinase and sodium fluoride on oxidation and phosphorylation by soybean mitochondria.[a]

Material added	Oxygen[b]			Phosphate[c]			P:O		
	S[d]	P[d]	K[d]	S	P	K	S	P	K
None	13.9	9.0	9.1	5.8	7.6	8.5	0.42	0.84	0.93
Hexokinase (2.5 mg/flask)	14.3	8.9	8.3	11.2	11.5	12.2	0.78	1.18	1.47
Na F (10^{-2} M)	9.3	6.3	6.2	--	--	6.3	--	--	1.01
Hexokinase + Na F	10.8	5.9	6.8	8.6	6.8	9.5	0.79	1.15	1.40

[a]All flasks contained substrate, 0.02 M; AMP, 0.0005 M; $MgSO_4$, 0.001 M; glucose, 0.1 M; sucrose, 0.1 M; phosphate, 0.00625 M. Malate, 0.0017 M, added with pyruvate.

[b]Oxygen uptake expressed as microatoms/hour/flask.

[c]Phosphate uptake expressed as micromoles/hour/flask.

[d]Succinate, pyruvate, ketoglutarate.

were obtained with succinate, pyruvate, and ketoglutarate, respectively. These ratios were raised to 0.8, 1.2, and 1.5 by the addition of 2.5 mg hexokinase per flask. The addition of fluoride, by itself or with hexokinase, had little effect on P:O ratios. Both oxygen uptake and phosphate disappearance were inhibited to about the same extent by the concentration of fluoride used, 10^{-2} M. These results were surprising in the light of data in the literature on the effect of fluoride. Some inhibition of oxygen uptake (20 per cent) was noted by Bonner and Millerd (16) upon the addition of 10^{-2} M fluoride, but the P:O ratio was increased considerably. Since it is assumed that ATP is being formed from ADP and AMP during phosphorylation, such an increase would be expected if fluoride were acting to inhibit ATPase activity, as has been demonstrated (51). Preliminary experiments, however, indicated that the ATPase activity of soybean mitochondria was too low to release a measurable amount of inorganic phosphate from 5 x 10^{-4} M ATP in 30 minutes at 30°C. This might account for the inability of added fluoride to increase the P:O ratios of this system with the concentration of adenylate used in these experiments.

Since 10^{-2} M sodium fluoride was found to be a strong inhibitor of oxygen uptake by soybean mitochondria in the presence of all substrates tested, the effect of various lower concentrations was investigated. Average results from three experiments of this type are presented in Table 9.

Succinate, ketoglutarate, and pyruvate oxidation were all slightly inhibited at concentrations of fluoride as low as 10^{-4} M. Effects of concentrations of fluoride lower than 10^{-2} M on phosphorylation and P:O ratios were not investigated.

Table 9. Effects of various concentrations of sodium fluoride on oxygen uptake by soybean mitochondria.[a]

Na F (M)	Oxygen uptake, % control		
	Succinate	Ketoglutarate	Pyruvate
1×10^{-2}	77	59	70
5×10^{-3}	79	76	90
1×10^{-3}	91	84	93
5×10^{-4}	90	90	-
1×10^{-4}	92	88	93
5×10^{-5}	97	94	-
1×10^{-5}	95	96	-

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M. Malate, 0.0017 M; added with pyruvate.

Variation within and between experiments

Results of numerous experiments indicated that there was much more variability between the activities of different enzyme preparations than there was within an experiment.

This is shown in Table 10 which presents data for oxygen uptake by duplicate flasks in several experiments. In eleven experiments with succinate as substrate, the mean difference between duplicates was 4.3 per cent, with a standard deviation of 3.7 per cent. With pyruvate as substrate the mean difference between duplicate flasks was 2.5 per cent, with a standard deviation of 3.9 per cent. Because of this close agreement between duplicates, it was decided to use only one flask per treatment in each experiment. Each treatment was repeated at least three times with different particle preparations, and treatment effects were calculated as average percentages of control rather than as $Q_{O_2}(N)$ values. This was done because of the relatively large variation in the ability of the particles of different preparations (experiments) to oxidize substrate. This variation is shown in Table 11, as is the relative ability of particles in different experiments to oxidize succinate, pyruvate, and ketoglutarate. Mean values for pyruvate, ketoglutarate, and endogenous oxidation were 41, 39, and 15 per cent, respectively, of the mean value for succinate oxidation. Although the average activity of soybean mitochondria with respect to pyruvate and ketoglutarate oxidation was about the same, relatively large variations may be seen in the ability of a given particle preparation to oxidize these two substrates. For example, the rate of ketoglutarate oxidation varied from approximately 65 per cent of the rate of pyruvate oxidation

Table 10. Agreement between duplicate flasks within experiments.[a]

Experiment	Substrate	Q_{O_2} (N)			
		Flask # 1	Flask # 2	Difference	Mean
1	Succinate	340	390	50	365
2	Succinate	291	282	9	287
3	Succinate	320	324	4	322
4	Succinate	307	298	9	303
5	Succinate	477	498	19	487
6	Succinate	413	407	6	410
7	Succinate	397	384	13	391
8	Succinate	460	468	8	464
9	Succinate	428	434	6	431
10	Succinate	625	590	35	608
11	Succinate	401	432	31	417
Mean		405	410	17.5	408
Standard Deviation					15.3
12	Pyruvate	241	200	14	207
13	Pyruvate	139	142	3	141
14	Pyruvate	150	149	1	150
15	Pyruvate	233	233	0	233
16	Pyruvate	170	175	5	173
17	Pyruvate	230	230	0	230
18	Pyruvate	234	223	11	229
Mean		196	193	4.9	195
Standard Deviation					7.6

[a]All flasks contained substrate, 0.02 M; ATP 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate 0.05 M. Malate, 0.0017 M, added with pyruvate.

Table 11. Effect of different substrates on oxygen uptake by soybean mitochondria.[a]

Experiment	Q_{O_2} (N)				
	Succinate (1)	Pyruvate (2)	Ketoglutarate (3)	Endog. (4)	Ratio 1:2:3:4
1	352	181	-	55	100:51:--:16
2	370	115	-	72	100:31:--:19
3	560	233	154	63	100:42:28:11
4	454	195	130	76	100:43:29:17
5	406	152	200	65	100:37:49:16
6	400	188	216	45	100:47:54:11
7	326	121	161	52	100:37:49:16
8	464	-	122	73	100:--:26:16
Mean	417	169	164	63	100:41:39:15

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M. Malate, 0.0017 M, added with pyruvate.

in two experiments shown in Table 11 to approximately 130 per cent in two others. Thus different soybean particle preparations varied both in their general level of oxidizing activity and in their relative ability to oxidize individual substrates.

Effect of Herbicides and Related Chemicals on Respiration of Soybean Mitochondria

2,4-Dichlorophenoxyacetic acid (2,4-D)

The effects of 2,4-D on mitochondrial enzyme systems were investigated in various ways. In addition to the standard procedure of adding a series of concentrations to the enzyme mixture and determining gas exchange, this chemical was also tested on hypocotyl segments in vitro. Also, soybean seedlings were sprayed before isolation of the mitochondria and changes in the oxidation and phosphorylation ability of the particles were observed. The effect of 2,4-D added directly to the flask on oxidative phosphorylation was likewise studied.

Effect on oxygen uptake and carbon dioxide evolution. Various concentrations of 2,4-D were added directly to the reaction mixture in Warburg flasks and oxygen uptake and carbon dioxide evolution were measured with succinate, pyruvate or ketoglutarate as substrate. The results of several experiments of this type are summarized in Table 12. Each figure is the average of at least three experiments.

High concentrations of 2,4-D inhibited both oxygen uptake and carbon dioxide evolution in the presence of all substrates tested, with oxygen uptake being inhibited to the greatest extent. With succinate as substrate, concentrations as low as 5×10^{-4} M brought about some inhibition of oxidation. The pyruvate and ketoglutarate systems were little affected by concentrations below 2×10^{-3} M. Endogenous respiration was unaffected by all levels of 2,4-D. No concentration of 2,4-D tested stimulated the oxidative activity of soybean mitochondria under the experimental conditions (Table 12). However, stimulation of oxygen uptake by soybean mitochondria with ketoglutarate as substrate was obtained if the phosphate concentration of the reaction mixture was lowered. Table 13 contains data comparing the results of typical experiments in which no inorganic phosphate or 5×10^{-4} M inorganic phosphate was added to the reaction mixture, with the average results of the experiments in which 5×10^{-2} M inorganic phosphate was added. It may be seen that oxygen uptake was stimulated by certain concentrations of 2,4-D in the presence of either 5×10^{-4} M or zero added inorganic phosphate. Stimulation as high as 50 per cent has been obtained. Not only was stimulation greatest in the latter system, but also inhibition by the highest level of 2,4-D used, 5×10^{-3} M, was much reduced in comparison with that of the systems to which inorganic phosphate had been added. Determination of inorganic phosphate in the reaction mixture

Table 12. Effect of 2,4-D on oxygen uptake and carbon dioxide evolution by soybean mitochondria.[a]

2,4-D (M)	Gas exchange, % control					
	Succinate		Pyruvate		Ketoglutarate	
	O_2	CO_2	O_2	CO_2	O_2	CO_2
1×10^{-2}	22	45	8	25	24	61
5×10^{-3}	24	45	24	32	56	75
2×10^{-3}	39	55	44	74	82	-
1×10^{-3}	59	90	98	92	89	83
5×10^{-4}	84	87	94	-	101	-
1×10^{-4}	97	96	100	91	99	88
1×10^{-5}	100	-	97	100	100	-
1×10^{-6}	104	-	105	91	100	-

[a]All flasks contained substrate, 0.02 M; ATP, 0.005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M. Malate, 0.0017 M, added with pyruvate.

Table 13. Effects of 2,4-D on oxygen uptake by soybean mitochondria in the presence of various quantities of inorganic phosphate in the reaction mixture.[a]

2,4-D (M)	Oxygen uptake, % control		
	Inorganic phosphate added (M)		
	0	5×10^{-4}	5×10^{-2}
5×10^{-3}	84	35	24
1×10^{-3}	110	94	89
5×10^{-4}	121	114	101
1×10^{-4}	115	-	99
5×10^{-5}	110	110	100
1×10^{-5}	115	106	100
Control, Q_{O_2} (N)	(95)	(127)	(195)

[a]All flasks contained ketoglutarate, 0.02 M; AMP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M.

at the end of the experiments in which zero inorganic phosphate was added showed that the flasks to which 2,4-D had been added were higher in phosphate than the controls. Similar stimulation of oxygen uptake and appearance of inorganic phosphate in the presence of 2,4-D have been demonstrated by Brody (17) with animal mitochondria in low phosphate systems.

Oxygen uptake was considerably reduced in the low phosphate controls in comparison to systems to which higher concentrations of inorganic phosphate had been added (Table 13). Since oxygen uptake by these low phosphate systems was stimulated by 2,4-D, it would appear that this herbicide was acting either to replace or release inorganic phosphate. The observation that the flasks to which 2,4-D was added contained more inorganic phosphate at the end of the experiment than the controls, indicated that 2,4-D was breaking down some organic phosphate compound. Thus, 2,4-D seemed to act as an uncoupling agent in these mitochondrial systems.

Since it has been shown that 2,4-D is more effective on plant tissue when used in a slightly acid solution (55), the effects of various pH levels on the response of soybean mitochondria to different concentrations of this herbicide were investigated. Average results from three experiments of this kind with succinate as substrate are presented in Table 14. Oxygen uptake was inhibited to a greater extent by 10^{-3} M 2,4-D at pH's above 7.0 than at those below this

level. Activity of the control, however, was also much lower at pH 7.4 than at pH 6.6, so the greater 2,4-D effects at the higher pH's may have been due to a general increased sensitivity of the particles. It seemed unlikely that the results could be explained on the basis of dissociation of the 2,4-D molecule, since at all the pH's used almost complete dissociation would be expected. Lower pH's were not used because of the rapid drop in oxidation of succinate under acid conditions (Figure 8).

Most studies of the effect of 2,4-D on plant respiration have been carried out with intact plants or with plant parts such as oat coleoptiles (38), bean stems (69), or pea stems (38). In such investigations, inhibition of respiration by high concentrations of 2,4-D and stimulation by low concentrations have been demonstrated. The results of a limited study of the effect of 2,4-D on the respiration of segments of etiolated soybean hypocotyls are presented in Table 15. Little change in respiration was brought about by the concentrations of 2,4-D used. It is surprising that 8×10^{-3} M had such little effect since Smith (69) found that approximately 5×10^{-4} M 2,4-D inhibited respiration of kidney bean stems 80 per cent, and Kelly and Avery (38) showed that 4×10^{-3} M 2,4-D inhibited respiration of pea stems 40 per cent. However, these data also indicate the variability of plant respiratory response to 2,4-D, so perhaps the results with soybean hypocotyl may be explained in terms of

Table 14. Effect of pH of reaction mixture on inhibition of succinate oxidation by 2,4-D.[a]

2,4-D (M)	Oxygen uptake, % control			
	7.4	7.1 pH	6.6	6.4
1×10^{-2}	22	-	-	15
5×10^{-3}	44	24	31	37
1×10^{-3}	54	63	92	82
5×10^{-4}	76	84	103	98
1×10^{-4}	103	100	101	95
5×10^{-5}	100	100	-	-
Control, Q_{O_2} (N)	(111)	(295)	(380)	(368)

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.1 M; phosphate, 0.05 M.

Table 15. Effect of *in vitro* treatment with 2,4-D on oxygen uptake by soybean hypocotyl segments.[a]

2,4-D (M)	Oxygen uptake[b]	Oxygen uptake, % control
Control	238	100
8×10^{-3}	227	95
4×10^{-3}	221	93
8×10^{-4}	255	107
4×10^{-4}	238	100

[a]All flasks contained hypocotyl segments, 0.25 ml 2,4-D and/or water to 2.0 ml.

[b]Oxygen uptake expressed as microliters O_2/hr/g fresh weight.

this variability.

Effect of pretreatment of hypocotyls on respiration. Experiments were also carried out to test the effect of spraying etiolated soybean plants with 2,4-D at various times before harvest on the activity of the isolated mitochondria. Table 16 shows the results of representative experiments of this type, in which soybeans were sprayed with 5×10^{-4} M 2,4-D at 24, 18, and 12 hours prior to harvest. The 24-hour and 18-hour treatments both increased the activity of particles in relation to the activity of particles from untreated plants of the same age. The 12-hour treatment affected mitochondrial activity in various ways, depending on the substrate used. Succinate oxidation was increased, pyruvate decreased, and ketoglutarate apparently unaffected by this treatment. In general, phosphate uptake was increased to about the same extent as oxygen consumption, although considerably more was consumed in the reaction mixture containing particles from the 12-hour treated plants with succinate or ketoglutarate as substrate.

The question arises whether 2,4-D changed the activity of the mitochondrial enzymes in vivo, or whether the increased activity may be related to changes in the morphology of the tissue as a whole. All sprayed plants were swollen and turgid at time of harvest, with the degree of such effects depending upon length of time since spraying. The 12-hour treated plants were least changed in appearance and particles

from these plants showed inconsistent respiratory responses to added substrate. Perhaps particles from turgid tissue did not lose as much activity during isolation or inhibitors were present in smaller quantities. Endogenous oxygen uptake was

Table 16. Effect of pretreatment of soybean hypocotyls with 0.0005 M 2,4-D, at various times before harvest, on the respiration and phosphorylation of isolated mitochondria.[a]

Treatment, hours before harvest	Substrate	Percentage of control		
		O_2 uptake	P uptake	P:O
24	Succinate	149	155	104
	Pyruvate	135	172	125
	Ketoglutarate	120	137	114
18	Succinate	132	133	100
	Pyruvate	137	156	113
	Ketoglutarate	134	106	75
12	Succinate	127	220	173
	Pyruvate	77	82	107
	Ketoglutarate	99	156	158

[a]All flasks contained substrate, 0.02 M; AMP, 0.0005 M; $MgSO_4$, 0.001 M; glucose, 0.1 M; sucrose, 0.1 M; phosphate, 0.00625 M. Malate, 0.0017 M, added with pyruvate.

also higher in experiments in which plants were pretreated with 2,4-D. This also would seem to indicate that the effect of the herbicide in increasing mitochondrial respiration was involved with changes in the tissue so that more-active

particles were isolated, rather than with changes in the mitochondrial complex of enzymes itself. Some support for this idea was provided by the results of experiments in which DCP (10^{-5} M) was sprayed on soybean hypocotyls 24 hours before isolation of mitochondria. This concentration of DCP brought about no change in the external appearance of the plants and decreased oxygen uptake of isolated mitochondria slightly (10 per cent) in relation to the control. If the respiratory effects of 2,4-D shown in Table 16 were brought about by changes in mitochondrial enzymes in vivo, similar results would be expected with DCP which has been shown to act in much the same way as 2,4-D on the respiration of intact tissue (25). Thus it would appear that mitochondria isolated from 2,4-D-sprayed plants are more active than from unsprayed plants, mainly because formative effects of this chemical lead to isolation of particles that have retained more of their original activity.

Effects on oxidative phosphorylation. The results of a representative experiment in which the effects of various concentrations of 2,4-D on both oxidation and phosphorylation of succinate were studied are presented in Table 17. As in earlier work on phosphorylation (Table 8) the disappearance of inorganic phosphate from the reaction mixture during the course of the experiment was considered to be an estimate of the number of high-energy phosphate bonds formed. Oxygen uptake is expressed as microatoms per hour and phosphate

disappearance as micromoles per hour.

Table 17. Effect of 2,4-D on oxidative phosphorylation by soybean mitochondria with succinate as substrate.[a]

2,4-D (M)	O_2 uptake[b]	P uptake[c]	P:O	Percentage of control		
				O_2	P	P:O
Control	13.5	12.8	0.94	100	100	100
5×10^{-3}	3.6	1.3	0.36	26	10	38
1×10^{-3}	6.6	3.5	0.53	49	27	57
5×10^{-4}	10.4	8.5	0.75	77	66	80
1×10^{-4}	12.0	11.8	0.98	88	92	104
5×10^{-5}	13.4	12.3	0.92	99	96	98

[a]All flasks contained substrate, 0.02 M; AMP, 0.0005 M; $MgSO_4$, 0.001 M; glucose, 0.1 M; sucrose, 0.1 M; phosphate, 0.00625 M; hexokinase, 2.5 mg.

[b]Oxygen uptake expressed as microatoms/hr.

[c]Phosphate uptake expressed as micromoles/hr.

Both oxygen and phosphate uptake were markedly inhibited by concentrations of 2,4-D of 5×10^{-4} M and higher, with phosphate uptake showing the greater effect. This resulted in a depression of the P:O ratio at these levels of 2,4-D treatment. Apparently the enzymes involved in phosphorylation *in vitro* were more severely affected by 2,4-D than those involved in oxidation. Similar results have been obtained by Brody (17) using animal mitochondria.

2,4-Dichlorophenol (DCP)

It has been demonstrated that some effects brought about by 2,4-D treatment were actually caused by DCP present as a contaminant (29). Since relatively high concentrations of 2,4-D were used in the present study, the possibility was considered that DCP might be involved in the results obtained. The 2,4-D used, as well as 2,4-D from other sources, was therefore tested for phenol content by the method of Folin and Ciocalteau (24). Average results from two determinations are presented in Table 18. It may be seen that both the Baker 2,4-D and the Eastman 2,4-D contained 0.04 per cent DCP. This meant that the highest concentration of 2,4-D used in any experiment, 0.01 M, contained 4×10^{-6} M DCP. The average results of experiments in which various concentrations of DCP were tested for effect on oxygen uptake by mitochondria, presented in Table 19, show that 4×10^{-6} M DCP had no affect on the oxidation of any of the substrates tested. It was assumed, therefore, that results obtained from 2,4-D treatments in this study were caused by the 2,4-D and not by DCP present as a contaminant. The possibility remains that 2,4-D was metabolized to DCP before it brought about these results.

At the highest concentration tested, DCP inhibited pyruvate oxidation completely, ketoglutarate oxidation about 80 per cent, and succinate oxidation only about 50 per cent. At 1.25×10^{-4} M, however, pyruvate oxidation was inhibited

Table 18. Dichlorophenol content of various samples of 2,4-D.

Source of 2,4-D	DCP content, mole percentage[a]
Commercial (Dupont sodium salt)	0.53
Baker "Purified" sodium salt	0.04
Sodium salt prepared from Eastman 2,4-D acid	0.04
American Chemical and Paint "Pure" sodium salt	0.14

[a]Total phenols present were determined and assumed to be all DCP.

Table 19. Effect of dichlorophenol on oxygen uptake by soybean mitochondria.[a]

DCP (M)	Oxygen uptake, % control			
	Substrate			
	Succinate	Pyruvate	Ketoglutarate	Endogenous
7.5×10^{-4}	41	0	21	-
5.0×10^{-4}	50	0	22	31
1.25×10^{-4}	46	74	66	-
1.0×10^{-4}	81	103	70	56
5×10^{-5}	101	100	75	-
1×10^{-5}	101	100	95	94
5×10^{-6}	103	103	100	-
1×10^{-6}	103	109	100	100

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.1 M; phosphate, 0.05 M. Malate, 0.0017 M, added with pyruvate.

only 25 per cent, ketoglutarate about the same, and succinate still about 50 per cent. Such results, in which pyruvate oxidation was inhibited to a greater extent than succinate at the highest concentration of added chemical, but less affected by lower concentrations, were also obtained with 2,4-D (Table 12). In contrast to the effects of 2,4-D and of all other chemicals tested, DCP inhibited endogenous respiration at the higher levels used.

Limited experiments carried out in connection with studies of 2,4-D and phosphorylation, indicated that DCP at 0.0001 M and 0.00005 M was a potent uncoupling agent (Table 20). The P:O ratio was reduced to 55 per cent and 60 per cent of the control by these concentrations. DNP was also tested for comparison with DCP and 2,4-D (Table 20). As expected, DNP inhibited phosphate disappearance at all concentrations tested while increasing oxygen uptake at the lower concentrations. At 10^{-4}M, the highest level tested, DNP almost completely inhibited phosphate disappearance and reduced oxygen uptake only about 30 per cent.

The effect of DCP on oxygen uptake by segments of soybean hypocotyls is shown in Table 21. It may be seen that the higher concentrations used inhibited oxygen uptake somewhat, but not to the extent that similar concentrations inhibited uptake by isolated mitochondria (Table 19). The The DCP in the experiments with hypocotyls was, of course, not in direct contact with the respiratory enzymes as it was

Table 20. Effects of dinitrophenol and dichlorophenol on oxidation and phosphorylation by soybean mitochondria with succinate as substrate.[a]

DNP (M)	O_2 uptake, microatoms/hr	P uptake, micromoles/hr	P:O
Control	9.8	13.9	1.40
5×10^{-6}	11.8	10.8	0.92
1×10^{-5}	14.8	10.8	0.73
5×10^{-5}	7.1	4.7	0.66
1×10^{-4}	7.0	0.3	0.04
DCP (M)			
Control	9.8	13.9	1.40
5×10^{-5}	11.8	9.9	0.84
1×10^{-4}	9.5	7.5	0.79

[a]All flasks contained succinate, 0.02 M; AMP, 0.0005 M; $MgSO_4$, 0.001 M; glucose, 0.1 M; hexokinase, 2.5 mg; NaF, 0.01 M; sucrose, 0.1 M; phosphate, 0.00625 M.

Table 21. Effect of dichlorophenol on oxygen uptake by soybean hypocotyl-segments treated *in vitro*.[a]

DCP (M)	Oxygen uptake[b]	Oxygen uptake, % control
Control	196	100
1×10^{-6}	213	109
1×10^{-5}	213	109
5×10^{-5}	203	104
1×10^{-4}	182	93
1×10^{-3}	141	72

[a]All flasks contained hypocotyl-segments, 0.25 ml 2,4-D and/or water to 2.0 ml.

[b]Oxygen uptake expressed as microliters O_2/hr/g fresh weight.

when added to mitochondrial suspensions. A slight stimulation was brought about by the lowest concentration tested. These results are similar to those obtained by French and Beevers (25) with corn coleoptile tissue, except that stimulations were not as great as those obtained by these investigators.

Indoleacetic acid (IAA)

The "natural" plant growth-substance IAA has been shown to affect the respiration of intact tissues in a manner similar to that of the herbicide 2,4-D. Experiments were conducted to see if the response of isolated soybean mitochondria to IAA was also similar to the response to 2,4-D. The results of such experiments are presented in Table 22, where each figure is the average of at least three experiments.

High concentrations of IAA inhibited oxygen uptake slightly with either succinate or pyruvate as substrate, but there was little effect below 10^{-3} M. Similar results were obtained by Dow (22) using lupine mitochondria, although he observed stimulation by 10^{-4} M IAA and no such stimulation was evident here in the presence of added substrate. However, slight stimulation of endogenous oxygen uptake was brought about by all concentrations of IAA tested. Since 10^{-9} M IAA stimulated endogenous oxygen uptake to as great an extent as 10^{-3} M, it is difficult to explain this increased oxidation in terms of the presence of indoleacetic acid oxidase. This

stimulation, however, does appear to be peculiar to IAA, since it was not consistently observed in comparable experiments with other chemicals. For example, 2,4-D slightly stimulated endogenous oxidation in a few experiments but inhibited it in just as many, with all effects within the limits of experimental error. Dow (22) also observed stimulation of endogenous oxygen uptake by IAA and attributed it to

Table 22. Effects of indoleacetic acid on oxygen uptake and carbon dioxide evolution by soybean mitochondria.[a]

IAA (M)	Gas exchange, % control			
	Succinate		Pyruvate	
	O_2	CO_2	O_2	CO_2
5×10^{-3}	73	104	88	84
1×10^{-3}	86	100	96	75
5×10^{-4}	96	-	-	-
1×10^{-4}	101	107	98	96
1×10^{-5}	98	103	96	87
1×10^{-6}	101	-	96	88
1×10^{-7}	103	-	94	-
1×10^{-8}	100	-	103	-
1×10^{-9}	105	-	100	-

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M. Malate, 0.0017 M, added with pyruvate.

oxidation of the IAA. This explanation appeared justified when he tested concentrations no lower than 10^{-4}M, but cannot

be used here where 10^{-9} M had as great an effect as the higher concentrations. Further study of this problem is necessary before a satisfactory explanation can be offered.

2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)

The results of experiments in which 2,4,5-T was added to Warburg flasks containing soybean mitochondria are presented in Table 23. Oxygen uptake and carbon dioxide evolution were determined with succinate or pyruvate as substrate. The data are averages of four experiments.

Table 23. Effect of 2,4,5-trichlorophenoxyacetic acid on oxygen uptake and carbon dioxide evolution by soybean mitochondria.[a]

2,4,5-T (M)	Gas exchange, % control			
	Succinate		Pyruvate	
	O_2	CO_2	O_2	CO_2
5×10^{-3}	18	83	12	54
1×10^{-3}	46	103	43	70
5×10^{-4}	75	-	64	-
1×10^{-4}	105	97	94	92
1×10^{-5}	103	100	88	95
1×10^{-6}	106	-	98	107
1×10^{-7}	100	-	96	-

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M. Malate, 0.0017 M, added with pyruvate.

In general, the results are similar to those obtained with 2,4-D (Table 12), except that 2,4,5-T seemed to be more

toxic at 5 x 10^{-4}M, especially with pyruvate as substrate. As with 2,4-D, carbon dioxide evolution was apparently not as severely inhibited as oxygen uptake, and no significant stimulation was obtained at any concentration of the herbicide tested. Endogenous oxygen uptake was unaffected by 2,4,5-T.

This similarity between the effects of 2,4-D and 2,4,5-T on plant respiration has been shown with intact tissues (50, 76) and with isolated lupine mitochondria (22). In the latter investigation, Dow found that 2,4,5-T at 10^{-4}M stimulated oxygen uptake with pyruvate as substrate, but inhibited succinate oxidation. Apparently mitochondria from different plants differ appreciably in their response to these herbicides.

2,2,Dichloropropionic acid (DCPA)

Since little information has appeared on the effect of DCPA on plant respiration, experiments were set up in which various concentrations of this herbicide were added to Warburg flasks containing segments from etiolated soybean hypocotyls. The results of a representative experiment of this type are presented in Table 24. Concentrations of DCPA above 10^{-2}M inhibited oxygen uptake slightly. Results in general were similar to those obtained with comparable concentrations of 2,4-D (Table 15).

The effects of DCPA on mitochondrial respiration were next investigated and are presented in Table 25. Each figure

Table 24. Effect of *in vitro* treatment with dichloropropionic acid on oxygen uptake by soybean hypocotyl segments.[a]

DCPA (M)	Oxygen uptake[b]	Oxygen uptake, % control
Control	229	100
2.6×10^{-1}	176	77
8.0×10^{-2}	191	85
2.6×10^{-2}	205	90
2.6×10^{-3}	221	97
1.3×10^{-3}	223	98
1.3×10^{-4}	242	106

[a]All flasks contained hypocotyl segments, 0.25 ml DCPA and/or water to 2.0 ml.

[b]Oxygen uptake expressed as microliters O_2/hr/g fresh weight.

Table 25. Effect of dichloropropionic acid on oxygen uptake and carbon dioxide evolution by soybean mitochondria.[a]

DCPA (M)	Gas exchange, % control			
	Succinate		Pyruvate	
	O_2	CO_2	O_2	CO_2
1×10^{-1}	22	-	36	100
5×10^{-2}	60	-	42	108
2×10^{-2}	72	-	75	80
1×10^{-2}	80	77	80	75
1×10^{-3}	95	95	92	96
1×10^{-4}	95	92	100	105
1×10^{-5}	95	95	98	94
1×10^{-6}	100	100	102	108

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M. Malate, 0.0017 M, added with pyruvate.

in this table is the average of at least three experiments. It may be seen that oxygen uptake by soybean mitochondria was inhibited by concentrations of DCPA above 10^{-3} M with either succinate or pyruvate as substrate. There was little difference between the effects on the different substrates. As with 2,4-D, carbon dioxide evolution was less affected than oxygen uptake. Endogenous oxygen uptake was unchanged by any concentration of DCPA tested.

Comparing data in Table 24 with that in Table 25, it appears that oxygen uptake by soybean mitochondria was more sensitive to DCPA than oxygen uptake by soybean hypocotyl segments. This has been found with 2,4-D (Tables 12 and 15) and seems reasonable, since one would expect a lower concentration than that applied to reach reactive sites in tissue segments. Thus, the same concentrations of added chemical would bring about less pronounced responses of segments than of isolated mitochondria.

Sodium chlorate

The effects of sodium chlorate on oxygen uptake and carbon dioxide evolution by soybean mitochondria are shown in Table 26. Only extremely high concentrations (0.5 M and 0.1 M) brought about appreciable changes. The highest concentration tested inhibited oxygen uptake with succinate as substrate more than with pyruvate as substrate. Carbon dioxide evolution with pyruvate as substrate was increased by both 0.5 M and 0.1 M sodium chlorate. Perhaps the herb-

icide was acting as an oxidizing agent at these concentrations. This high oxidizing ability of the chlorate ion has been offered as a possible explanation of the herbicidal action of sodium chlorate (2).

Table 26. Effects of sodium chlorate on oxygen uptake and carbon dioxide evolution by soybean mitochondria.[a]

$NaClO_3$ (M)	Gas exchange, % control			
	Succinate		Pyruvate	
	O_2	CO_2	O_2	CO_2
5×10^{-1}	27	105	75	157
1×10^{-1}	88	-	90	128
5×10^{-2}	82	108	82	105
1×10^{-2}	96	119	101	100
1×10^{-3}	104	100	110	114
1×10^{-4}	99	93	100	100
1×10^{-5}	105	118	111	117
1×10^{-6}	107	97	100	107

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M. Malate, 0.0017 M, added with pyruvate.

Since only high concentrations of sodium chlorate affected soybean mitochondria, it seemed possible that ionic strength of the solution might be an important factor. The neutral salts, sodium chloride and potassium chloride, were therefore tested at comparable concentrations. The data

in Table 27 are averages of four experiments in which sodium chloride was used. They show that sodium chloride inhibited oxygen uptake as severely as did sodium chlorate, although there was no stimulation of carbon dioxide evolution. In fact, carbon dioxide evolution was inhibited by 0.5 M sodium chloride, although not as severely as oxygen uptake. Similar results were obtained when potassium chloride was added at the same concentrations as in Table 27.

Table 27. Effects of sodium chloride on oxygen uptake and carbon dioxide evolution by soybean mitochondria.[a]

NaCl (M)	Gas exchange, % control			
	Succinate		Pyruvate	
	O_2	CO_2	O_2	CO_2
5×10^{-1}	10	91	50	68
1×10^{-1}	54	-	84	104
1×10^{-2}	91	100	89	100
1×10^{-3}	105	95	103	88
1×10^{-4}	101	110	100	90
1×10^{-5}	102	-	106	-

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M. Malate, 0.0017 M, added with pyruvate.

The inhibition of oxygen uptake by high salt concentrations did not, therefore, appear to be connected with a particular ion. Possibly 0.5 M salt made the solution sufficiently

hypertonic that damage to the mitochondria resulted. Including the sucrose and phosphate present, the total molarity of the high salt reaction mixture approached 0.8 M. No information on tonicity as high as 0.8 M has been found in the plant mitochondrial literature, but salt solutions even more concentrated than this have been used in the isolation of animal mitochondria with little or no loss of activity (67). In spite of this, injury to the soybean mitochondria by the high salt concentrations used may probably be best explained in terms of hypertonicity.

Trichloroacetate (TCA)

Average results from three experiments in which TCA was used are presented in Table 28. At high concentrations TCA

Table 28. Effects of trichloroacetic acid on oxygen uptake and carbon dioxide evolution by soybean mitochondria.[a]

TCA (M)	Gas exchange, % control			
	Succinate		Pyruvate	
	O_2	CO_2	O_2	CO_2
1×10^{-1}	15	264	9	97
5×10^{-2}	30	190	50	88
1×10^{-2}	84	109	111	103
1×10^{-3}	96	90	113	98
1×10^{-4}	91	95	100	75
1×10^{-5}	94	-	105	98
1×10^{-6}	91	-	107	86

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M. Malate, 0.0017 M, added with pyruvate.

was found to inhibit strongly the uptake of oxygen by mitochondria in the presence of either succinate or pyruvate. Carbon dioxide evolution was strongly stimulated by high concentrations of TCA with succinate as substrate, but relatively unaffected with pyruvate as substrate. There appeared to be a slight stimulation of oxygen uptake with pyruvate as substrate by 10^{-2} M and 10^{-3} M TCA.

TCA is often used as a protein precipitant and is probably acting in this manner at the highest concentrations used. Since mitochondria contain a high percentage of protein, this reaction would account for the strong inhibition of oxygen uptake at 0.1 M. No explanation is offered for the effects on carbon dioxide evolution by these concentrations, except for the possibility that TCA is decarboxylating the substrate.

Herbicidal oil

Small quantities of a herbicidal oil (Standard L-8764) were added, with and without a wetting agent (B-1956), to Warburg flasks containing soybean mitochondria. The effects of these treatments on oxygen uptake and carbon dioxide evolution with pyruvate as substrate are presented in Table 29. All data are averages of at least two experiments. Both oxygen uptake and carbon dioxide evolution were inhibited by the oil alone, but greater inhibition was obtained by the addition of the wetting agent. However, the wetting agent used alone inhibited respiration as much as when mixed with

the oil, probably because of disorganization of the lipid fraction of the mitochondria by this material. Since quantities used in these experiments were much larger than

Table 29. Effects of a herbicidal oil (Standard L-8764) and a wetting agent (B-1956) on oxygen uptake and carbon dioxide evolution by soybean mitochondria with pyruvate as substrate.[a]

Treatment	Gas exchange, % control	
	O_2 uptake	CO_2 evolution
Oil-1.6%	50	38
Oil-3.3%	60	87
Oil-6.6%	48	81
Oil-1.6% + wetting agent-1.6%	30	19
Oil-3.3% + wetting agent-1.6%	23	53
Oil-6.6% + wetting agent-1.6%	26	62
Wetting agent-1.6%	33	46
Wetting agent-3.3%	17	41

[a]All flasks contained pyruvate, 0.02 M; malate, 0.0017 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M.

one would expect to find in the cells of treated plants, this dissolving action of the oil and wetting agent on isolated mitochondria is probably not duplicated in plant cells.

Maleic hydrazide

Data from representative experiments with succinate or pyruvate as substrate, presented in Table 30, indicate that oxygen uptake by soybean mitochondria was affected only

Table 30. Effects of maleic hydrazide on oxygen uptake by soybean mitochondria.[a]

Maleic hydrazide (M)	Oxygen uptake, % control	
	Succinate	Pyruvate
1×10^{-2}	91	75
5×10^{-3}	99	94
1×10^{-3}	114	88
5×10^{-4}	102	88
1×10^{-4}	105	100
5×10^{-5}	100	-
1×10^{-5}	102	96
5×10^{-6}	99	-

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M. Malate, 0.0017 M, added with pyruvate.

slightly by any of the concentrations of maleic hydrazide tested. These results are similar to those obtained by other workers using intact plant tissue (39, 56).

Since maleic hydrazide has been shown to be capable of reversing inhibition effects on plant growth by high concentrations of auxin (45), its effect on 2,4-D-induced inhibition of oxygen uptake by isolated soybean particles was

studied. These experiments showed that concentrations of maleic hydrazide from 10^{-4}M to 5 x 10^{-3}M did not change the inhibition of oxygen uptake of soybean mitochondria brought about by 5 x 10^{-3}M 2,4-D.

2,3,5-Triiodobenzoic acid (TIBA)

This chemical, which has been called an auxin synergist (45), was tested with soybean mitochondria both alone (Table 31) and in mixtures with 2,4-D (Table 32). Data in both tables are averages from three experiments. Used alone, TIBA inhibited oxygen uptake with succinate as substrate at concentration as low as 5 x 10^{-5}M. This chemical, therefore, was the most active inhibitor of oxidation of any tested, although it had little effect on carbon dioxide evolution except at concentrations of 10^{-3}M or higher. Mixtures of TIBA and 2,4-D inhibited oxygen uptake slightly more than either chemical used alone (Table 32). These results are compatible with the findings of others that TIBA is an auxin synergist (26, 45).

Coumarin

Although coumarin has been shown to bring about formative effects similar to those of 2,4-D (4), it did not resemble this herbicide in its effect on oxygen uptake by soybean mitochondria (Table 33). The data in this table are averages from three experiments, and indicate that coumarin had no effect on oxygen uptake with succinate or pyruvate as substrate at any concentration tested.

Table 31. Effects of triiodobenzoic acid on oxygen uptake and carbon dioxide evolution by soybean mitochondria with succinate as substrate.[a]

TIBA (M)	Gas exchange, % control	
	O_2 uptake	CO_2 evolution
1.25×10^{-3}	14	74
1×10^{-3}	20	80
5×10^{-4}	15	101
1×10^{-4}	60	92
5×10^{-5}	86	102
1×10^{-5}	100	107
5×10^{-6}	103	104
1×10^{-6}	101	112
1×10^{-7}	100	-
1×10^{-8}	102	-

[a]All flasks contained succinate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M.

Table 32. Effects of triiodobenzoic acid, 2,4-D, and mixtures on oxygen uptake by soybean mitochondria with succinate as substrate.[a]

TIBA (M)	2,4-D (M)	Oxygen uptake, % control
0	10^{-2}	22
0	10^{-3}	58
0	10^{-4}	97
1×10^{-4}	0	60
1×10^{-4}	10^{-4}	52
5×10^{-5}	0	86
5×10^{-5}	10^{-4}	72
1×10^{-5}	0	100
1×10^{-5}	10^{-4}	91
1×10^{-6}	0	101
1×10^{-6}	10^{-4}	90
1×10^{-7}	0	102
1×10^{-7}	10^{-4}	97

[a]All flasks contained succinate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.02 M; phosphate, 0.05 M.

Mixtures of coumarin and 2,4-D decreased succinate oxidation much more than either used alone (Table 34). This synergism was more pronounced than that shown by TIBA and 2,4-D (Table 32). These results show that coumarin and 2,4-D have a synergistic response in in vitro mitochondrial systems similar to that shown in intact tissues (45).

Table 33. Effects of coumarin on oxygen uptake by soybean mitochondria.[a]

Coumarin (M)	Oxygen uptake, % control	
	Succinate	Pyruvate
2×10^{-3}	97	98
1×10^{-3}	94	98
5×10^{-4}	98	100
1×10^{-4}	98	96
1×10^{-5}	98	93

[a]All flasks contained substrate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M; phosphate, 0.05 M. Malate, 0.0017 M, added with pyruvate.

Table 34. Effects of mixtures of coumarin and 2,4-D on oxygen uptake by soybean mitochondria with succinate as substrate.[a]

2,4-D (M)	Oxygen uptake, % control		
	Coumarin (M)		
	0	1×10^{-3}	2×10^{-3}
0	100	98	97
1×10^{-4}	100	94	84
5×10^{-4}	91	82	72

[a]All flasks contained succinate, 0.02 M; ATP, 0.0005 M; $MgSO_4$, 0.001 M; sucrose, 0.2 M,

DISCUSSION

The original interest in this research was the characterization of some of the physiological actions of herbicides. Since herbicides are known to have varying stimulating and depressing effects on respiration, the effects of various chemicals on the particulate oxidizing systems of soybean hypocotyl cells were investigated. No work has been reported on the isolation or action of soybean mitochondria. The properties of these particles were, therefor, investigated for comparison with results with other material.

Mitochondria isolated from mung beans have been well characterized by other investigators (16, 51, 52, 53). Since the results of experiments using mung bean particles in the present study agreed closely with their observations, it was considered that any differences between results obtained with soybean mitochondria and those reported in the literature with other plant particles were probably due to differences in the plant material rather than to major experimental differences.

In general, the oxidative activity of soybean particles was similar to that of particles from mung beans. The various Krebs cycle intermediates tested were all oxidized, although fumarate and oxaloacetate were oxidized only slowly. Since the Krebs cycle enzymes were apparently

present, one would expect other substrates as well as pyruvate to be oxidized through the entire cycle. If such were the case, the differences in the relative rates of oxidation pointed out above would not be expected. Millerd et al. (53) and Dow (22) also found that fumarate oxidation was much slower than the oxidation of other intermediates, but offered no explanation. Perhaps endogenous fumarate produced by the oxidation of succinate is more readily oxidized than exogenous fumarate because of better access to the enzyme complex. Even though this endogenous fumarate is oxidized at a greater rate than exogenous fumarate, its further metabolism must proceed less rapidly than succinate oxidation, judging from the r.q. of 0.3 obtained consistently for succinate. A similar r.q. for succinate oxidation by particles from Arum maculatum was obtained by Hackett and Simon (32), indicating that a large part of this substrate is apparently not completely oxidized in these isolated enzyme systems. On the other hand, the r.q. of 1.3 obtained with pyruvate as substrate indicates that this material is completely oxidized to carbon dioxide and water. This is in agreement with the findings of Millerd et al. (53) using mung bean mitochondria. Soybean mitochondria oxidized keto-glutarate with an average r.q. of 1.2, which is close to the value obtained by Hackett and Simon (32) using particles from Arum maculatum, and perhaps not significantly below the theoretical value for complete oxidation.

Oxygen uptake by isolated soybean particles was greatly reduced when either sucrose or phosphate was omitted from the grinding or suspending solution. Considering these results in the light of observations by others (6, 22, 40, 41, 42, 51, 52, 53), it is apparent that plant particles must be kept immersed in a slightly hypertonic solution to maintain a high level of activity. Data from the present experiments in which high concentrations of sodium chloride or potassium chloride were added to soybean particle preparations, indicate that injury may also result from too high tonicity. The optimum level seems to be about 0.4 M in the grinding medium.

Although the necessity for added sucrose or mannitol in the suspending solution has been recognized by all investigators, the same is not true for phosphate. Laties (40, 41, 42) and Tager (72, 73) did not add phosphate to the sucrose solutions in which mitochondria were isolated from cauliflower buds and Avena coleoptiles. The increased activity of soybean particles in the presence of added phosphate was similar to the results of Millerd (51) with mung beans, Dow with white lupines (22), Hackett and Simon (32) with Arum maculatum, and Beaudreau and Remmert (6) with kidney beans. Millerd's experiments (51) indicated that the added inorganic phosphate acted to inhibit phosphorolytic breakdown of some essential substance or substances. If this is the correct explanation, there must be a greater tendency

for this breakdown to occur in particles isolated from some plants, including soybeans, than from others.

Most investigators have subjected mitochondrial suspensions to one or more washings during the course of the procedure (22, 40, 51, 72). Millerd (51) and Laties (40) reduced endogenous oxygen uptake to a negligible amount by one washing, but Dow (22) and Tager (72) were unable to do so. Similar difficulty was encountered in the present study, where endogenous oxygen uptake of once-washed particles varied from about 10 to 20 per cent of oxygen uptake in the presence of succinate. This endogenous activity could be reduced by further washing, but the activity of pyruvate oxidizing enzymes was also markedly reduced by more than one washing. The single washing which was used routinely, however, not only decreased endogenous oxygen uptake about 50 per cent, but also doubled succinate oxidation and tripled pyruvate oxidation. Apparently both endogenous substrate and some interfering substance or inhibitors of succinate and pyruvate oxidation were removed. The decrease in pyruvate oxidation with more than one washing was probably due to a loss of enzymatic activity over the longer periods of time involved between initial isolation and addition to the Warburg flasks. The succinoxidase system, which did not show this dropping off of activity, is apparently more stable.

The pH optimum for soybean particle activity was demonstrated to be about 6.9, which is only slightly lower than

that used by other investigators (40, 53, 72). In general, cofactor requirements were also similar to those of particles from mung beans (51, 53), cauliflower buds (40, 41, 42), Avena coleoptiles (72), and kidney beans (6), although a strong cytochrome c response was obtained, which differs from the results of Millerd et al. (53) and Tager (72). Millerd et al. (53) relate the necessity for added cytochrome c to the method of preparation, since they were able to produce mung bean particles that did respond to this cofactor by omitting or lowering the concentration of phosphate in the grinding medium. Since phosphate was used in the present study and other steps in the isolation procedure were similar to those of Millerd et al. (53), it would seem that cytochrome c saturation of isolated particles is influenced by the species of plant from which the particles are taken as well as by the method of isolation.

Soybean mitochondria have been shown to take up inorganic phosphate during oxidation and presumably to esterify it into high-energy phosphate bonds, as has been observed with particles from other plants (16, 42, 51, 53). In the present study, phosphate disappearance was enhanced by the addition of hexokinase. In the presence of glucose, hexokinase mediates the transfer of high-energy phosphate from ATP to glucose-6-phosphate. A receptor molecule for phosphate esterification is thus liberated and the glucose-hexokinase acts as a trapping system for all phosphate subsequently

esterified. Bonner and Millerd (16) obtained no increase in P:O ratios by adding hexokinase, and stated that mung bean hexokinase is firmly bound to the mitochondria and does not limit the phosphorylation reaction. This apparently is not the case with soybean mitochondria. In this respect, they resemble cauliflower bud mitochondria, since Laties (40) found that the latter particles did not contain enough hexokinase to permit maximum oxidation. However, P:O ratios obtained with soybean particles (about 1.0) were closer to those obtained with mung beans (16) than to those of Laties (43) with cauliflower. It would appear that the phosphorylative enzymes of both mung beans and soybeans are more easily damaged during preparation than those of cauliflower. Probably the true P:O ratios of these plants are considerably higher than 1.0, perhaps even approaching the efficiency of mammalian mitochondria (P:O ratio 3-4).

The various chemicals tested on soybean mitochondria in this study acted alike in certain ways. With the exception of coumarin, all of them inhibited oxygen uptake by soybean mitochondria when added in relatively high concentrations to the reaction mixture. This suggests an osmotic effect, and probably such an explanation is correct for chemicals that inhibited only at concentrations of 0.1 M or higher, such as sodium chlorate. Others, including 2,4-D and 2,4,5-T, were inhibitory down to 5×10^{-4} M; and TIBA, the most active chemical tested in this respect, inhibited oxygen uptake at

5×10^{-5}M. Inhibition in these cases must have been caused by a toxic action of the added chemical other than through a change in tonicity of the medium. This toxic action of most of the chemicals is apparently not specific, since oxygen uptake was affected to approximately the same extent in the presence of all substrates tested. Such a generalization may not hold true for 2,4-D, where it appeared that succinate oxidation was slightly more sensitive than either pyruvate or ketoglutarate oxidation. This exception, however, is not sufficiently striking to keep one from concluding that, in general, chemicals that inhibit oxygen uptake by isolated soybean mitochondria, at concentrations too low to be injurious through osmotic action, do so by a general toxic action on the whole enzyme complex rather than on individual enzymes. A similar conclusion was arrived at by Dow (22), on the basis of his results with growth substances on isolated lupine mitochondria.

Another similarity between the effects of the various chemicals was that oxygen uptake was usually more strongly inhibited than carbon dioxide evolution. Similar results have been obtained with intact tissues (35). The explanation suggested (35, 70), that the aerobic phase of respiration was more sensitive to 2,4-D than the anaerobic phase, cannot be used here since only the aerobic system is considered to be located in plant mitochondria (52). The present results would be obtained if oxidation of substrate was more severely inhibited than decarboxylation. Although no direct expe-

rimental evidence is available, this interpretation would appear to be sound.

Of the various chemicals tested, 2,4-D was investigated most extensively, as there seemed to be more reason to believe that it might affect aerobic respiration (15, 35, 70). With the usual amount of inorganic phosphate in the reaction mixture (0.00625 M), both oxygen uptake and phosphate disappearance were inhibited by added 2,4-D. The greater inhibition of the latter process provides some verification for the hypothesis of Bonner and Bandurski (15) that herbicides act through changes in the phosphate transfer system. When little or no inorganic phosphate was added to the reaction mixture, oxygen uptake by the control system was much reduced. The lack of phsophate may have reduced oxygen uptake because of reduced phosphorylation, or because phosphate is necessary to maintain general mitochondrial activity. Under these conditions of low phosphate, oxygen uptake was stimulated by concentrations of 2,4-D from 10^{-5} M to 10^{-3} M and the usually strong inhibitory action of 5×10^{-3} M 2,4-D was greatly reduced. Limited data indicated that the inorganic phosphate content of the flasks to which 2,4-D had been added was considerably higher than the controls at the end of the experiment. Thus, it would appear that 2,4-D stimulated oxygen uptake under conditions of low inorganic phosphate by splitting off phosphate from organic phosphate compounds. In other words, 2,4-D seemed to act as an uncoupling agent

in these systems.

Similar results have been obtained by Brody (17) with rat liver mitochondria. However, he obtained a maximum stimulation of oxygen uptake that was over 100 per cent of the low phosphate control. In the present study the maximum increase obtained in a single experiment was 50 per cent of the control and the usual increase was about 20 per cent. With intact bean plants, Loustalot et al. (48) found that large amounts of inorganic phosphate accumulated in bean plants as the toxic action of 2,4-D set in. Thus, it would appear that 2,4-D may affect the phosphate transfer system both in vitro and in vivo.

In plant tissue it has generally been considered that auxins (including 2,4-D) stimulate respiration by mediating an energy transfer step leading toward growth, and in this way bring about an increased supply of high-energy phosphate acceptors (14, 15, 45). Under this scheme, respiratory stimulation would precede growth stimulation. The other alternative, that respiratory stimulation results from growth stimulation has been championed by French and Beevers (25) who showed that respiration was promoted by some substances that did not promote growth, such as DNP and DCP. According to these investigators, auxins increase respiration by bringing about growth through utilization of high-energy phosphate, whereas chemicals like DNP and DCP increase respiration by freeing phosphate acceptors but without

utilizing the energy thus released in growth. In particle preparations from soybeans where growth cannot take place, 2,4-D seemed to be acting more like an uncoupling agent (as DCP, DNP) than like an auxin. Further study to compare the effects of such growth substances as 2,4-D, 2,4,5-T, and IAA would be of interest.

In intact tissue 2,4-D is known to behave as an auxin. If it is assumed that the major difference between the aerobic respiratory system of intact cells and of isolated mitochondria is the coupling of this system to growth *in vivo*, the present study has provided some verification for the hypothesis that auxins stimulate respiration by stimulating growth and not the reverse. Otherwise stimulation of mitochondrial respiration by 2,4-D *in vitro* would be expected. One must, of course, consider possible differences in the concentration of 2,4-D at the site of action *in vivo* and *in vitro*. Whether this herbicide acts to stimulate respiration by promoting growth or by acting as an uncoupling agent may largely depend on the concentration present.

Various limitations in the use of isolated mitochondria in the study of herbicidal action became apparent during the course of this investigation. The large variation between the activities of particles used in different experiments made comparisons of the effects of herbicides difficult. This was partially overcome by expressing the data in each experiment as percentages of the control. The figures thus

obtained were averaged for several experiments. In this method it is assumed that a chemical has the same effect on highly active particles as on those that are less active. This assumption seemed to be valid, since the effects of a given concentration of chemicals in different experiments, expressed as percentages of control, usually agreed fairly closely, even though the controls might differ widely.

Interpretation of the results obtained with isolated mitochondria in terms of intact tissue is difficult because of the vastly different surroundings in which the particles are acting. In addition, it is extremely difficult to determine the concentration of chemical that actually is present in a treated cell. Thus, when a certain quantity of chemical gives a particular effect when added to isolated soybean particles, the question arises concerning the concentration that should be applied to an intact plant to give a comparable quantity in the cytoplasm. For these reasons, little attempt was made to make quantitative comparisons between results obtained by treating isolated mitochondria with those in which intact tissues were treated. Nevertheless, it was considered that the reactions of isolated particles to various chemicals would be qualitatively comparable to the effects of these herbicides on mitochondrial enzymes <u>in vivo</u>.

Although the results of this study were disappointing in that the site of action of none of the herbicides tested

was pin-pointed, a general inhibition of the respiratory enzymes of isolated soybean mitochondria was demonstrated. In relation to the inhibition brought about by 2,4-D, TIBA was considerably more active, 2,4,5-T slightly more so, and IAA, DCPA, and maleic hydrazide slightly less active. All other compounds tested were effective only at very high concentrations. It would appear, therefore, that of the chemicals tested, those known to have growth regulating properties when applied to intact plants or plant segments were the more active respiratory inhibitors in vitro.

Since 2,4-D has been shown to affect oxidative phosphorylation, an investigation of the relationship between other herbicides and phosphorylation would be of interest. Further work on the uncoupling action of 2,4-D in comparison with the effects of DCP and DNP in both high and low phosphate systems should also be done. Such investigations coupled with studies on intact tissue should lead to a better understanding of the mechanism of herbicide action.

SUMMARY

Active particles, presumed to be mitochondria, have been isolated by differential centrifugation from the hypocotyls of etiolated soybean seedlings. These particles were capable of oxidizing all Krebs cycle intermediates tested, although fumarate and oxaloacetate were oxidized relatively slowly. R.q. values indicated that most of these intermediates were oxidized completely to carbon dioxide and water. However, succinate oxidation proceeded consistently with a r.q. of about 0.3, indicating that a large proportion of this substrate was incompletely oxidized.

Oxygen uptake in the presence of succinate, pyruvate or ketoglutarate was increased two to four times by the addition of ATP, $MgSO_4$, and cytochrome c. Maximum activity was obtained when both sucrose and phosphate were added to the grinding and suspending media, and when the reaction mixture was buffered near pH 7.0. Endogenous oxidation was reduced 50 per cent by one washing and at the same time the oxidation of exogenous substrate was increased two to three times.

Average P:O ratios of 0.4, 0.8, and 0.9 were obtained without added hexokinase or fluoride for succinate, pyruvate, and ketoglutarate, respectively. These ratios were increased to 0.8, 1.2, and 1.5 by the addition of exogenous hexokinase. In all cases, sodium fluoride decreased oxygen uptake 30 to

40 per cent without affecting P:O ratios. Dinitrophenol and dichlorophenol were shown to uncouple phosphorylation from ketoglutarate oxidation.

All herbicides and related chemicals that were tested were found to inhibit oxidation at high concentrations. Some of these effects at concentrations above 0.1 M were probably brought about through osmotic changes. In most cases, however, a general toxic effect of the chemical on mitochondrial enzymes was indicated. TIBA was the most active inhibitor of oxidation tested. In general, none of the chemicals tested stimulated respiration in the presence of added substrate. One exception was the stimulation of oxygen uptake by 2,4-D in systems containing low added inorganic phosphate.

The herbicide 2,4-D was investigated more extensively than any other chemical. The effects of pretreatment and *in vitro* treatment with 2,4-D were studied in relation to phosphate uptake as well as oxidation. Both processes were inhibited *in vitro*, with phosphorylation inhibited to the greater extent. Pretreatment of hypocotyls with 5×10^{-4} M 2,4-D increased the oxidative and phosphorylative ability of isolated particles. This increased activity was probably related to changes in the morphology of the tissue, leading to isolation of more-active particles, rather than to changes in the mitochondrial complex of enzymes *in vivo*. Analysis of the 2,4-D for DCP contamination showed that the

results obtained with 2,4-D could not have been caused by the low concentration of DCP present.

All of the chemicals tested appeared to inhibit *in vitro* respiration by means of a general inhibition of mitochondrial enzymes rather than by a specific effect on certain enzymes. While quantitative comparisons between the effects of various concentrations of chemical on plant particles *in vitro* and *in vivo* are difficult, it is considered that the results obtained by treatment with the various chemicals are qualitatively comparable.

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